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Use of dose-dependent absorption into target tissues to more accurately predict cancer risk at low oral doses of hexavalent chromium

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ABSTRACT

The mouse dose at the lowest water concentration used in the National Toxicology Program hexavalent chromium (CrVI) drinking water study (NTP, 2008) is about 74,500 times higher than the approximate human dose corresponding to the 35-city geometric mean reported in EWG (2010) and over 1000 times higher than that based on the highest reported tap water concentration. With experimental and environmental doses differing greatly, it is a regulatory challenge to extrapolate high-dose results to environmental doses orders of magnitude lower in a meaningful and toxicologically predictive manner. This seems particularly true for the low-dose extrapolation of results for oral CrVI-induced carcinogenesis since dose-dependent differences in the dose fraction absorbed by mouse target tissues are apparent (Kirman et al., 2012). These data can be used for a straightforward adjustment of the USEPA (2010) draft oral slope factor (SFo) to be more predictive of risk at environmentally-relevant doses. More specifically, the evaluation of observed and modeled differences in the fraction of dose absorbed by target tissues at the point-of-departure for the draft SFo calculation versus lower doses suggests that the draft SFo be divided by a dose-specific adjustment factor of at least an order of magnitude to be less over-predictive of risk at more environmentally-relevant doses.

1. Introduction

In recent years, there has been a great deal of scientific debate and new research regarding exactly how and under what conditions CrVI is likely to induce cancer following oral exposure (e.g., Thompson et al., 2011a; McCarroll et al., 2010; USEPA, 2010). Some significant topics of debate concern issues relevant to the mode of action (MOA) and whether the excess risk observed at very high mouse oral doses of CrVI would be expected to extrapolate downward to significantly lower, truly environmentally-relevant human doses in a linear manner or if a nonlinear/threshold dose–response should be expected at such low doses. Such topics include the roles of mutagenicity and chronic hyperplasia in CrVI-induced carcinogenicity in target tissues, if the MOA and/or gastrointestinal (GI) extracellular reductive capacity likely impart a nonlinear/threshold character to the dose–response, and the potential that mouse oral doses in NTP (2008) exceeded the extracellular CrVI reductive capacity of the stomach/GI tract.

As part of the CrVI MOA research project (e.g., Thompson et al., 2011a), Proctor et al. (2012) report that stomach reducing capacity was likely exceeded at doses causing cancer in the mouse small intestine, and indicate that physiologically-based toxicokinetic (PBTK) models are necessary to account for competing kinetic rates in extrapolating target tissue dose for the purpose of risk assessment. If extracellular CrVI reductive capacity is exceeded at high drinking water concentrations such as those inducing cancer of the small intestine in NTP (2008), increased tissue uptake would be anticipated compared to lower doses (Thompson et al., 2011b). In other words, dose-dependent changes in the fraction of dose absorbed would be expected at doses which exceed stomach/GI extracellular CrVI reductive capacity compared to those that do not, with a higher dose fraction absorbed at doses exceeding reductive capacity.

In this study, tissue concentration data collected at various doses as part of the CrVI MOA research project (including some doses lower than those used in NTP, 2008) are evaluated to:

(1) quantify differences in the dose fraction absorbed at relevant doses; and
(2) derive factors based on dose-dependent changes in target tissue absorption that may be used to adjust the draft oral slope factor (SFo) to be more predictive of risk at lower, more environmentally-relevant doses.

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Table 1
Total chromium target tissue concentrations in B6C3F1 mice.[a]

Drinking water concentration (mg SDD/L)	Dose (mg Cr/kg-day)	Body weight[b] (g)	Total daily dose[c] (mg Cr/day)	Duodenum tissue concentration (mean mg Cr/kg tissue)	±SD	95% UCL[d] (mg Cr/kg tissue)	95% LCL[e] (mg Cr/kg tissue)	Jejunum tissue concentration (mean mg Cr/kg tissue)	±SD	95% UCL (mg Cr/kg tissue)	95% LCL (mg Cr/kg tissue)	Ileum tissue concentration (mean mg Cr/kg tissue)	±SD	95% UCL (mg Cr/kg tissue)	95% LCL (mg Cr/kg tissue)
0	0	25.8	0	0.017	0.007	0.022	0.012	0.046	0.044	0.078	0.014	0.020	0.01	0.027	0.013
0.3[f]	0.024	26.4	0.001	0.056	0.015	0.067	0.045	0.034	0.021	0.049	0.019	0.014	0.000	0.014	0.014
4	0.32	25.9	0.008	1.5	0.27	1.7	1.3	0.11	0.052	0.15	0.07	0.042	0.03	0.066	0.018
14	1.1	26.3	0.029	***7.3***	0.78	7.9	6.7	0.33	0.29	0.54	0.12	0.13	0.03	0.15	0.11
60	4.6	25.3	0.116	***33.5***	5.0	37.2	29.8	***4.7***	3.3	7.1	2.3	***0.92***	1.0	1.66	0.18
170	11.6	24.9	0.289	***42.4***	12.4	51.5	33.3	***21.6***	14.8	32.5	10.7	***1.8***	1.1	2.6	1.0
520	30.9	23.3	0.720	***60.9***	14.1	71.3	50.5	***13.9***	6.9	19.0	8.8	***2.3***	0.86	2.9	1.7

[a] Drinking water and tissue data taken from Table 3 of Kirman et al. (2012), who reported ***bold italicized*** values as significantly different than controls ($p < 0.05$).
[b] Body weight data from Table S2 of Thompson et al. (2011b).
[c] Calculated as mg Cr/kg-day × body weight in kilograms.
[d] 95%UCL = mean + (1.645 × SE) where SE = SD/n^0.5 and $n = 5$.
[e] 95%LCL = mean − (1.645 × SE) where SE = SD/n^0.5 and $n = 5$.
[f] Corresponds to the federal MCL of 0.1 mg Cr/L; MW of Cr_2/MW of SDD ≈ 104/298 ≈ 0.35 as conversion factor to convert SDD concentrations to Cr.

Table 2
Added chromium target tissue concentrations in B6C3F1 mice.[a]

Drinking water dose (mg Cr/kg-day)	Body weight[b] (g)	Total daily dose[c] (mg Cr/day)	Duodenum tissue concentration (mean added mg Cr/kg tissue)	±SD	95% UCL[d] (added mg Cr/kg tissue)	95% LCL[e] (added mg Cr/kg tissue)	Jejunum tissue concentration (mean added mg Cr/kg tissue)	±SD	95% UCL (added mg Cr/kg tissue)	95% LCL (added mg Cr/kg tissue)	Ileum tissue concentration (mean added mg Cr/kg tissue)	±SD	95% UCL (added mg Cr/kg tissue)	95% LCL (added mg Cr/kg tissue)
0.024	26.4	0.001	0.039	0.015	0.050	0.028	0	0.021	0	0	0	0.000	0	0
0.32	25.9	0.008	1.5	0.3	1.7	1.3	0.068	0.052	5.78E−05	1.62E−05	0.021	0.033	0.045	−0.003
1.1	26.3	0.029	7.2	0.8	7.8	6.6	0.28	0.29	2.72E−04	3.68E−05	0.11	0.03	0.13	0.09
4.6	25.3	0.116	33.5	5.0	37.2	29.8	4.7	3.3	3.79E−03	1.21E−03	0.9	1.0	1.6	0.16
11.6	24.9	0.289	42.4	12.4	51.5	33.3	21.5	14.8	1.69E−02	5.55E−03	1.8	1.1	2.6	1.0
30.9	23.3	0.720	60.9	14.1	71.3	50.5	13.8	6.9	9.24E−03	4.27E−03	2.3	0.9	3.0	1.6

[a] Drinking water doses and added Cr (over background) tissue data taken from Table 8 of Kirman et al. (2012) with background shown as zero added.
[b] Body weight data from Table S2 of Thompson et al. (2011b).
[c] Calculated as mg Cr/kg-day × body weight in kilograms.
[d] 95%UCL = mean + (1.645 × SE) where SE = SD/n^0.5 and $n = 5$.
[e] 95%LCL = mean − (1.645 × SE) where SE = SD/n^0.5 and $n = 5$.

Table 3
Absorbed dose fraction estimates for the mouse duodenum.

Drinking Water Concentration (mg SDD/L)	Dose[a] (mg Cr/kg-day)	Total Daily Dose (mg Cr/day)	Duodenum Fraction of Body Weight[b]	Duodenum Weight[c] (kg)	Duodenum Total Cr[d] (mg)	95% UCL Duodenum Total Cr (mg)	95% LCL Duodenum Total Cr (mg)	Mean Dose Fraction Absorbed[e]	95% UCL Dose Fraction Absorbed	95% LCL Dose Fraction Absorbed
0	0	0	0.012	3.10E-04	5.26E-06	6.86E-06	3.67E-06			
0.3[f]	0.024	0.001	0.012	3.17E-04	***1.77E-05***	2.12E-05	1.42E-05	1.97E-02	2.27E-02	1.67E-02
4	0.32	0.008	0.012	3.11E-04	4.66E-04	5.28E-04	4.04E-04	5.56E-02	6.29E-02	4.84E-02
14	*1.1*[g]	0.029	0.012	3.16E-04	***2.30E-03***	2.48E-03	2.12E-03	7.95E-02	8.57E-02	7.32E-02
60	4.6	0.116	0.012	3.04E-04	1.02E-02	1.13E-02	9.05E-03	8.73E-02	9.69E-02	7.78E-02
170	11.6	0.289	0.012	2.99E-04	1.27E-02	1.54E-02	9.94E-03	4.38E-02	5.33E-02	3.44E-02
520	30.9	0.720	0.012	2.80E-04	1.70E-02	1.99E-02	1.41E-02	2.36E-02	2.77E-02	1.96E-02

[a] Doses and total daily doses from Table 1.
[b] Tissue-specific fractions of body weight from Table 4 of Kirman et al. (2012).
[c] Calculated as fraction of body weight × body weight from Table 1.
[d] Calculated as tissue weight × tissue concentration (mean, 95% UCL, or 95% LCL) from Table 1; tissue concentrations associated with ***bold italicized*** values were compared to each other and are statistically significantly different by unpaired *t*-test ($p < 0.001$).
[e] Corrected for background concentrations in controls at 0 dose.
[f] Corresponds to the federal MCL of 0.1 mg Cr/L.
[g] Corresponds to the POD used for the draft SFo ($BMDL_{10}$ values of 1.0–1.2 mg/kg-day).

Table 4
Absorbed dose fraction estimates for the mouse jejunum.

Drinking Water Concentration (mg SDD/L)	Dose[a] (mg Cr/kg-day)	Total Daily Dose (mg Cr/day)	Jejunum Fraction of Body Weight[b]	Jejunum Weight[c] (kg)	Jejunum Total Cr[d] (mg)	95% UCL Jejunum Total Cr (mg)	95% LCL Jejunum Total Cr (mg)	Mean Dose Fraction Absorbed[e]	95% UCL Dose Fraction Absorbed	95% LCL Dose Fraction Absorbed
0	0	0	0.021	5.42E-04	2.49E-05	4.25E-05	7.39E-06			
0.3[f]	0.024	0.001	0.021	5.54E-04	***1.88E-05***	2.74E-05	1.03E-05	0	0	4.58E-03
4	0.32	0.008	0.021	5.44E-04	5.98E-05	8.06E-05	3.90E-05	4.21E-03	4.61E-03	3.82E-03
14	*1.1*[g]	0.029	0.021	5.52E-04	***1.82E-04***	3.00E-04	6.44E-05	5.44E-03	8.91E-03	1.97E-03
60	4.6	0.116	0.021	5.31E-04	2.50E-03	3.79E-03	1.21E-03	2.12E-02	3.22E-02	1.03E-02
170	11.6	0.289	0.021	5.23E-04	1.13E-02	1.70E-02	5.60E-03	3.90E-02	5.87E-02	1.94E-02
520	30.9	0.720	0.021	4.89E-04	6.80E-03	9.29E-03	4.32E-03	9.41E-03	1.28E-02	5.99E-03

[a] Doses and total daily doses from Table 1.
[b] Tissue-specific fractions of body weight from Table 4 of Kirman et al. (2012).
[c] Calculated as fraction of body weight × body weight from Table 1.
[d] Calculated as tissue weight × tissue concentration (mean, 95% UCL, or 95% LCL) from Table 1; tissue concentrations associated with ***bold italicized*** values were compared to each other and practically achieved a statistically significant difference by unpaired *t*-test ($p = 0.052$).
[e] Corrected for background concentrations in controls at 0 dose, negative corrected values set to zero.
[f] Corresponds to the federal MCL of 0.1 mg Cr/L.
[g] Corresponds to the POD used for the draft SFo ($BMDL_{10}$ values of 1.0–1.2 mg/kg-day).

Table 5
Absorbed dose fraction estimates for the mouse ileum.

Drinking Water Concentration (mg SDD/L)	Dose[a] (mg Cr/kg-day)	Total Daily Dose (mg Cr/day)	Ileum Fraction of Body Weight[b]	Ileum Weight[c] (kg)	Ileum Total Cr[d] (mg)	95% UCL Ileum Total Cr (mg)	95% LCL Ileum Total Cr (mg)	Mean Dose Fraction Absorbed[e]	95% UCL Dose Fraction Absorbed	95% LCL Dose Fraction Absorbed
0	0	0	0.0063	1.63E-04	3.25E-06	4.33E-06	2.17E-06			
0.3[f]	0.024	0.001	0.0063	1.66E-04	***2.33E-06***	2.33E-06	2.33E-06	0	0	2.43E-04
4	0.32	0.008	0.0063	1.63E-04	6.85E-06	1.08E-05	2.89E-06	4.35E-04	7.83E-04	8.65E-05
14	*1.1*[g]	0.029	0.0063	1.66E-04	***2.15E-05***	2.48E-05	1.82E-05	6.32E-04	7.09E-04	5.56E-04
60	4.6	0.116	0.0063	1.59E-04	1.47E-04	2.64E-04	2.94E-05	1.23E-03	2.23E-03	2.34E-04
170	11.6	0.289	0.0063	1.57E-04	2.82E-04	4.09E-04	1.55E-04	9.66E-04	1.40E-03	5.31E-04
520	30.9	0.720	0.0063	1.47E-04	3.38E-04	4.30E-04	2.45E-04	4.64E-04	5.92E-04	3.37E-04

[a] Doses and total daily doses from Table 1.
[b] Tissue-specific fractions of body weight from Table 4 of Kirman et al. (2012).
[c] Calculated as fraction of body weight × body weight from Table 1.
[d] Calculated as tissue weight × tissue concentration (mean, 95% UCL, or 95% LCL) from Table 1; tissue concentrations associated with ***bold italicized*** values were compared to each other and are statistically significantly different by unpaired *t*-test ($p < 0.001$).
[e] Corrected for background concentrations in controls at 0 dose, negative corrected values set to zero.
[f] Corresponds to the federal MCL of 0.1 mg Cr/L.
[g] Corresponds to the POD used for the draft SFo ($BMDL_{10}$ values of 1.0–1.2 mg/kg-day).

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2. Materials and methods

Tissue concentration data reported by Kirman et al. (2012) were evaluated for this study. Kirman et al. report total and added chromium (Cr) mouse target tissue (i.e., duodenum, jejunum, ileum) concentrations that were collected to support the rodent PBTK model (Tables 1 and 2). In addition to drinking water concentrations used in the CrVI rodent drinking water study (NTP, 2008), these data include two lower water concentrations (0.3 and 4 mg sodium dichromate dehydrate (SDD)/L) and their corresponding daily Cr doses. The lowest water concentration tested for Kirman et al. corresponds to the federal maximum contaminant level for chromium (MCL of 0.1 mg Cr/L), making these data more relevant to possible environmental exposures than those from the NTP study (although still at concentrations and doses much higher than typical human exposures). Additionally, the current study uses USEPA benchmark dose (BMD) software (version 2.5) to model tissue concentration versus dose so that absorbed dose fractions corresponding to doses up to three times lower than the lowest tested for Kirman et al. (2012) can be calculated.

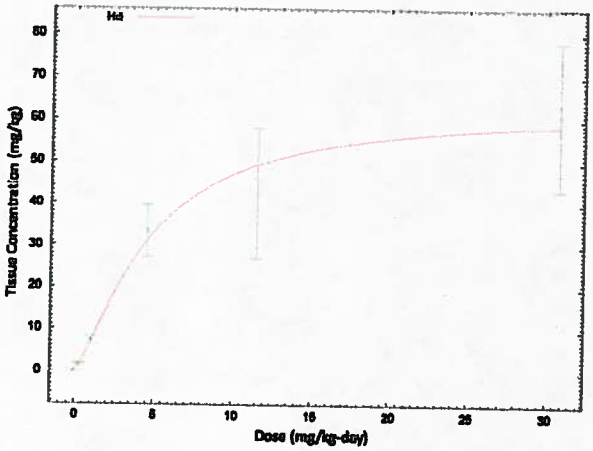

Fig. 1. Mouse duodenum tissue concentration versus daily dose.

The dose fractions absorbed into target tissues were calculated using the target tissue concentration data and tissue weights to first calculate the total amount of Cr in the target tissue (i.e., tissue concentration in mg Cr/kg tissue × tissue weight in kg = total mg Cr in tissue), and then dividing by the total daily dose (mg Cr/day, although use of cumulative dose would not change the relative differences in dose fraction absorbed at various doses). However, the target tissue concentration data presented in Table 1 for CrVI exposed mice are not corrected for the background Cr tissue levels present in control mice not exposed to CrVI. Thus, when calculating the CrVI dose fractions absorbed by these tissues (presented later in Tables 3–5), the background total Cr in a tissue was subtracted from that in exposed mice to represent only the additional Cr present in tissues due to the CrVI exposure (e.g., total Cr in a tissue due to CrVI exposure = total tissue Cr – background total tissue Cr in control mice). While this correction is not needed for the added Cr (over background) tissue concentration data presented in Table 2, data from both tables were used in order to evaluate and ensure consistency of results. The same process was used for 95% upper confidence limit (UCL) and 95% lower confidence limit (LCL) estimates. Accordingly, the dose fraction absorbed by a target tissue (i.e., duodenum, jejunum, ileum) at a given dose is calculated as follows:

Dose Fraction Absorbed = Total Added Cr in Target Tissue/Cr Dose

Absorbed dose fraction calculations based on the reported tissue concentration means, 95% UCL and 95% LCL estimates, and modeled tissue concentrations at even lower doses can be used to derive an adjustment factor for the draft SFo (0.5 per mg/kg-day; USEPA, 2010) to make it more predictive of excess risk at low doses, that is, doses lower than the point-of-departure (POD) used to calculate the SFo. More specifically, an evaluation of these tissue concentration data (based on both the empirical data collected and modeling the data) utilizing relatively straightforward calculations is used in this study to determine the factors by which the fractions of dose absorbed by target tissues decrease at lower, more environmentally-relevant doses compared to the POD made basis for the draft SFo ($BMDL_{10}$ values of 1–1.1 mg/kg-day). These factors account for dose-dependent changes in the dose fraction absorbed that are important to adjust for when the SFo is calculated based on a dose where an appreciably higher fraction is absorbed compared to the fractions absorbed at lower doses where the SFo will be used to estimate risk:

Adjustment Factor = DFA_{POD}/DFA_{ERD}

where:

DFA_{POD} = ∑ dose fractions absorbed by target tissues at the SFo POD; and DFA_{ERD} = ∑ dose fractions absorbed by target tissues at a lower, more environmentally-relevant dose where the SFo will be used to estimate risk.

Table 6
Duodenum best-fitting model tissue concentration prediction.

Hill Model (non-constant variance) equation:	*Y* [tissue conc. in mg/kg at dose] = intercept + *v** dose^*n*/(*k*^*n* + dose^*n*)
Parameters	Inputs
Dose (mg/kg-day)[a]	0.008
Intercept	0.018
v	62.397
n	1.406
k	4.638
Solve for *Y* [tissue conc. in mg/kg at dose]	**0.026**

[a] Corresponds to one-third the mouse dose at the federal MCL.

Table 7
Absorbed dose fraction estimates based on modeled tissue concentrations for the mouse duodenum.

Drinking water concentration (mg SDD/L)	Dose[a] (mg Cr/kg-day)	Total daily dose (mg Cr/day)	Duodenum tissue concentration[b] (mg Cr/kg tissue)	Duodenum total Cr[c] (mg)	Mean dose fraction absorbed[d]
1/3 the MCL	0.008	2.11E−04	0.026	8.19E−06	1.38E−02
1/2 the MCL	0.012	3.16E−04	0.032	1.02E−05	1.55E−02

[a] Doses and total daily doses at 1/3 and 1/2 the MCL were calculated based on these fractions × the doses at the MCL of 0.1 mg Cr/L (0.3 mg SDD/L) from Table 1.
[b] Tissue concentrations at 1/3 and 1/2 the MCL based on the BMD modeling equation in Table 6.
[c] Calculated as predicted tissue concentration x tissue weight at the MCL of 0.1 mg Cr/L (0.3 mg SDD/L) from Table 1.
[d] Corrected for the mean background duodenum tissue concentration in controls at 0 dose (0.017 mg Cr/kg tissue or total tissue Cr of 5.26E−06 mg) from Table 1.

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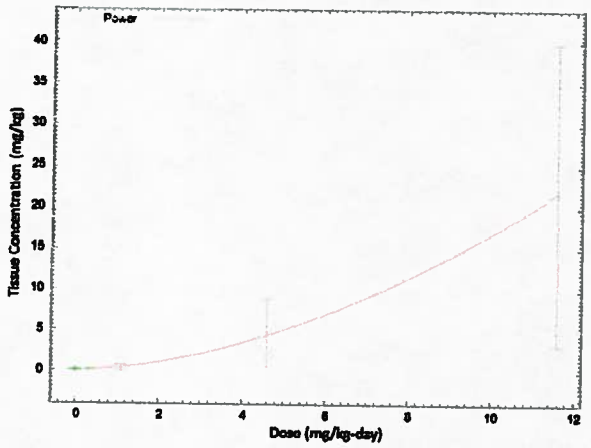

Fig. 2. Mouse jejunum tissue concentration versus daily dose.

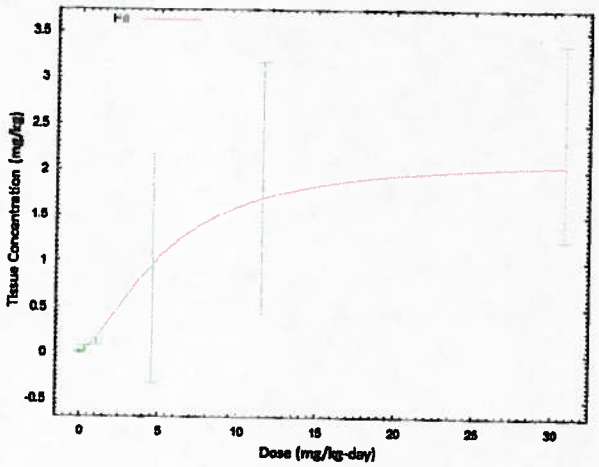

Fig. 3. Mouse ileum tissue concentration versus daily dose.

The draft SFo can simply be divided by this factor to be more predictive of risk a dose lower and more environmentally-relevant than those used in NTP (2008):

Adjusted SFo = SFo/Adjustment Factor

Lastly, examples of adjusted USEPA draft SFo values for CrVI are used to calculate excess risk at the federal MCL (0.1 mg/L) and a high but environmentally-relevant drinking water concentration (i.e., the maximum reported city drinking water concentration in EWG, 2010).

3. Results and discussion

There are dose-dependent differences in the dose fraction absorbed by target tissues (Tables 3–5) based on analysis of the tissue concentration data collected to support the rodent PBTK model (Kirman et al., 2012). As drinking water concentrations and associated doses increase from 0 to 60 mg SDD/L, the mean dose fractions absorbed also increase. This is true for all three target tissues, including the duodenum as the most carcinogenesis-responsive tissue (Table 3) and the jejunum (Table 4) as the secondary contributor to the draft SFo.

The dose fractions absorbed based on 95% UCL tissue concentrations also generally increase with dose as drinking water concentrations increase from 0 to 60 mg SDD/L. Based on 95% LCL tissue concentrations, the dose fractions absorbed by the duodenum (Table 3) also increase with dose over this drinking water concentration range (95% LCL results for the jejunum and ileum were more mixed).

While the dose fractions absorbed by target tissues increase with dose as drinking water concentrations increase from 0 to 60 mg SDD/L (Tables 3–5), it is apparent at the highest and least environmentally-relevant drinking water concentration doses (e.g., the two highest doses for the duodenum and ileum and the highest dose for the jejunum) that these tissues are unable to continue to absorb an ever-increasing fraction of the dose (although measured tissue concentrations are higher at these extremely high doses). Kirman et al. (2012) note that there is lower fractional absorption at the higher doses (>10 mg/kg-day at the two highest doses) where CrVI absorption is saturated, perhaps due to a toxic response (e.g., villi toxicity affecting transporter-mediated absorption and greater cell sloughing). However, as these higher doses are entirely irrelevant to environmental doses, this animal study high-dose phenomenon (CrVI absorption saturation at exceedingly high doses) does not detract from the significance of the results presented in the current paper for lower study doses that are still orders of magnitude higher than environmental exposures. For example, while the lowest water concentration tested in Kirman et al. of 0.3 mg SDD/L (0.1 mg Cr/L) is about 50 times less than the lowest concentration of 14.3 mg SDD/L tested in NTP (2008), it is still 555 times higher than the 35-city drinking water geometric mean (GM) and about 8 times higher than the city with the highest drinking water concentration (EWG, 2010).

In regard to more tissue- and dose-specific results, the duodenum and jejunum are the target tissues where the vast majority of adenomas/carcinomas were found in NTP (2008). Table 3 shows that the dose fraction absorbed by the mouse duodenum is approximately four times higher at the POD used for the draft SFo derivation than at the federal MCL (note that the duodenum tissue concentrations associated with these doses are statistically significantly different than each other; $p < 0.001$). This is significant given that the duodenum was the target tissue where most of

Table 8
Absorbed dose fraction estimates based on modeled tissue concentrations for the mouse jejunum and ileum.

Drinking water concentration (mg SDD/L)	Dose[a] (mg Cr/kg-day)	Total daily dose (mg Cr/day)	Jejunum tissue concentration[b] (mg Cr/kg tissue)	Jejunum total Cr[c] (mg)	Mean dose fraction absorbed[d]	Ileum tissue concentration[b] (mg Cr/kg tissue)	Ileum total Cr[c] (mg)	Mean dose fraction absorbed[d]
1/3 the MCL	0.008	2.11E−04	0.0434	2.406E−05	0	0.0164	2.73E−06	0
1/2 the MCL	0.012	3.16E−04	0.0435	2.410E−05	0	0.0165	2.74E−06	0

[a] Doses and total daily doses at 1/3 and 1/2 the MCL were calculated based on these fractions × the doses at the MCL of 0.1 mg Cr/L (0.3 mg SDD/L) from Table 1.
[b] Jejunum and ileum tissue concentrations at 1/3 and 1/2 the MCL based on the BMD modeling equations (not shown) from best-fitting models.
[c] Calculated as predicted tissue concentration × tissue weight at the MCL of 0.1 mg Cr/L (0.3 mg SDD/L) from Table 1.
[d] BMD model-predicted jejunum and ileum tissue concentrations at 1/3 and 1/2 the MCL were just below the control (0 dose) background tissue levels of 0.046 and 0.020 mg Cr/kg tissue, respectively, so to correct for background tissue concentrations the dose fraction absorbed values at 1/3 and 1/2 the MCL for the jejunum and ileum were set to zero.

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Table 9
Absorbed dose fraction estimates for the three mouse target tissues and SFo adjustment factors.

Drinking water concentration (mg SDD/L)	Dose[a] (mg Cr/kg-day)	Total daily dose (mg Cr/day)	Duodenum mean dose fraction absorbed[b]	Duodenum 95% UCL dose fraction absorbed	Duodenum 95% LCL dose fraction absorbed	Jejunum mean dose fraction absorbed[b]	Jejunum 95% UCL dose fraction absorbed	Jejunum 95% LCL dose fraction absorbed	Ileum mean dose fraction absorbed[b]	Ileum 95% UCL dose fraction absorbed	Ileum 95% LCL dose fraction absorbed	3-Tissue mean dose fraction absorbed[c]	3-Tissue 95% UCL dose fraction absorbed	3-Tissue 95% LCL dose fraction absorbed
1/3 MCL	0.008	2.11E−04	1.38E−02			0			0			**1.38E−02**		
1/2 MCL	0.012	3.16E−04	1.55E−02			0			0			1.55E−02		
0.3[d]	0.024	6.34E−04	1.97E−02	2.27E−02	1.67E−02	0	0	4.58E−03	0	0	2.43E−04	**1.97E−02**	**2.27E−02**	**2.15E−02**
4	0.32	8.29E−03	5.56E−02	6.29E−02	4.84E−02	4.21E−03	4.61E−03	3.82E−03	4.35E−04	7.83E−04	8.65E−05	6.03E−02	6.83E−02	5.23E−02
14	**1.1**[e]	2.89E−02	7.95E−02	8.57E−02	7.32E−02	5.44E−03	8.91E−03	1.97E−03	6.32E−04	7.09E−04	5.56E−04	**8.55E−02**	**9.53E−02**	**7.58E−02**
60	4.6	1.16E−01	8.73E−02	9.69E−02	7.78E−02	2.12E−02	3.22E−02	1.03E−02	1.23E−03	2.23E−03	2.34E−04	1.10E−01	1.31E−01	8.83E−02
170	11.6	2.89E−01	4.38E−02	5.33E−02	3.44E−02	3.90E−02	5.87E−02	1.94E−02	9.66E−04	1.40E−03	5.31E−04	8.38E−02	1.13E−01	5.43E−02
520	30.9	7.20E−01	2.36E−02	2.77E−02	1.96E−02	9.41E−03	1.28E−02	5.99E−03	4.64E−04	5.92E−04	3.37E−04	3.35E−02	4.11E−02	2.59E−02
									SFo adjustment factors[f]:					
									Based on lowest dose (at MCL)	**4.3**	4.2	3.5		
									Based on modeling at 1/3 MCL	**6.2**				

[a] Doses and total daily doses from Table 1, except doses at 1/3 and 1/2 the MCL were calculated based on these fractions × the doses at the MCL of 0.1 mg Cr/L (0.3 mg SDD/L).
[b] Mean and 95% UCL/LCL values from Tables 3–5 for doses tested in Kirman et al. (2012), and Tables 7 and 8 for mean dose fraction absorbed estimates at 1/3 and 1/2 the MCL.
[c] Sum of dose fractions absorbed (corrected for background tissue concentrations) for all three tissue mean, 95% UCL, or 95% LCL values.
[d] Corresponds to the federal MCL of 0.1 mg Cr/L.
[e] Corresponds to the POD used for the draft SFo ($BMDL_{10}$ values of 1.0–1.2 mg/kg-day).
[f] Calculated as dose fraction absorbed at draft SFo POD/fraction absorbed at the MCL or 1/3 the MCL.

the adenomas/carcinomas occurred in NTP (2008) and therefore was the principal contributor to the draft SFo. Similar to Table 3, Tables 4 and 5 show that the dose fractions absorbed into the mouse jejunum and ileum are higher at the POD used for the draft SFo derivation than at the federal MCL. This is of particular importance for the jejunum since this target tissue was a secondary contributor to the mouse adenomas/carcinomas observed in NTP (2008) and therefore contributed secondarily to the draft SFo. Not surprisingly, since the calculations in Tables 3–5 account for background tissue concentrations, essentially identical results were obtained using the added Cr tissue data provided in Table 2 (calculations not shown). Consequently, analyses based on data from Table 2 are not discussed further.

Greater differences in the dose fraction absorbed were found (compared to the dose fraction absorbed at the draft SFo POD) when duodenum tissue concentrations were modeled as a function of dose (BMD software version 2.5) in order to estimate absorbed dose fractions at one-half and one-third of the MCL. Fig. 1 shows good model fit (goodness-of-fit was evaluated by visual inspection with scaled residuals <|2| and a goodness-of-fit p value >0.1).

The equation and parameter estimates for this response function (provided by BMD software) were then used to calculate the estimated mean duodenum tissue concentrations at one-half and one-third of the MCL (3.2E–02 and 2.6E–02 mg/kg, respectively) since the MCL was the lowest water concentration for which data are provided in Kirman et al. (2012), drinking water concentrations are typically significantly below the MCL (e.g., EWG, 2010), and BMD modeling is generally not used to extrapolate to doses far below the experimental range (USEPA, 1995). For example, Table 6 provides the relevant inputs for the duodenum tissue concentration calculation at one-third of the federal MCL.

As in Table 3, these tissue concentrations and the total daily doses that would have been associated with one-half and one-third of the MCL (3.2E–04 and 2.1E–04 mg Cr/day, respectively) were used to estimate the dose fractions absorbed at these lower drinking water concentrations. The calculated absorbed dose fractions were approximately 1.6E–02 and 1.4E–02, respectively (Table 7).

Based on these results, the calculated dose fraction absorbed by the mouse duodenum is approximately six times higher at the POD used for the draft SFo derivation than at one-third of the federal MCL. This is significant given that most cancers occurred in this tissue in NTP (2008) and that typical drinking water concentrations are still almost 200 times lower than one-third the MCL (0.033 mg/L/35-city drinking water GM of 0.00018 mg/L ≈ 183).

Predictions by response function equations from good-fitting BMD models (Figs. 2 and 3) for the jejunum (high dose dropped) and ileum at even one-half the MCL (4.3E–02 and 1.6E–02 mg/kg, respectively) were slightly lower than mean background levels for those tissues (calculations not shown). Therefore, to account for background tissue concentrations, the dose fractions absorbed by these tissues (corrected for background) were set to zero at one-third and one-half of the MCL (Table 8).

Finally, in Table 9 absorbed doses by all three target tissues (from Tables 3–5, 7 and 8) are used to calculate overall SFo adjustment factors which account for differences in the dose fraction absorbed at the POD made basis for the draft SFo versus the fractions absorbed at lower, more environmentally-relevant doses (i.e., 1/3 the MCL and the lowest dose tested for Kirman et al., 2012).

The results in Table 9 show that the dose fraction absorbed by target tissues at the POD dose used in USEPA (2010) for the draft SFo calculation ($BMDL_{10}$ values of 1–1.1 mg/kg-day) is approximately four times higher than that at the MCL and about six times higher than that predicted at one-third of the MCL. Fig. 4 shows dose fraction absorbed versus dose for the lower drinking water concentrations of 0.3–60 mg SDD/L, which are closer to (although still significantly above) environmentally-relevant drinking water concentrations.

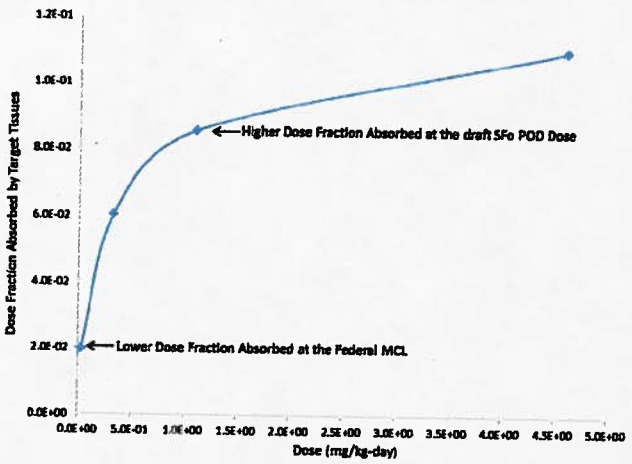

Fig. 4. Dose fraction absorbed versus dose.

Consideration of the shape of the curve in Fig. 4 suggests that using proportionality/linearity to estimate the expected dose fraction absorbed at truly low, environmentally-relevant water concentrations may be predictive. The 35-city drinking water GM (0.00018 mg/L) is over 500 times lower than the MCL (0.1 mg/L), the lowest water CrVI concentration tested in Kirman et al. (2012) and shown in Fig. 4. An estimate of the dose fraction absorbed at 0.00018 mg/L based on proportionality with the fraction absorbed at the lowest water concentration tested (0.3 mg SDD/L or 0.1 mg Cr/L) would be:

$$\text{Dose Fraction Absorbed}(\times)/0.00018\text{mgCr/L} = 1.97\text{E}-02/0.1\text{mgCr/L}$$

$$\text{Dose Fraction Absorbed}(\times) = (1.97\text{E}-02/0.1\text{mgCr/L}) \times 0.00018\text{mgCr/L}$$

$$\text{Dose Fraction Absorbed}(\times) = 3.55\text{E}-05$$

This estimate of the dose fraction which may be absorbed at the 35-city drinking water GM is over 2400 times lower than that calculated (8.55E–02 from Table 9) for the water concentration and dose (1.1 mg Cr/kg-day at 14 mg SDD/L) corresponding to the POD for the draft SFo (an adjustment factor of 2408). Even for the city with the highest drinking water concentration (0.0129 mg/L) reported in EWG (2010), the estimate of the dose fraction absorbed (2.54E–03; calculation not shown) is over 30 times lower than that calculated for the draft SFo POD (an adjustment factor of 34).

4. Conclusions

The above analyses show dose-dependent differences in the dose fraction absorbed by target tissues. More specifically, the dose fraction absorbed increases with dose from 0 to 60 mg SDD/L (0–21 mg Cr/L), which is up to 210 times the federal MCL (0.1 mg/L). Additionally, compared to the POD dose used in USEPA (2010) for the draft SFo calculation ($BMDL_{10}$ values of 1–1.1 mg/kg-day), analysis of the tissue concentration data collected (Table 9) indicates that the fractions of dose absorbed into target tissues of the mouse small intestine (duodenum, jejunum, ileum) are appreciably lower at lower doses. This may be due to dose-dependent changes

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in the competing rates of reduction/detoxification prior to CrVI absorption by target tissues. Based on both the absorbed dose fractions calculated using measured target tissue concentration data and the absorbed fractions predicted at doses lower than those tested, it is further concluded that the magnitude of risk over-estimation by the draft SFo (0.5 per mg/kg-day) increases as it is used to estimate excess risk at progressively lower, more environmentally-relevant water concentrations where the dose fractions absorbed become progressively lower.

To be more predictive of risk, the draft SFo for CrVI should be adjusted by dose-specific adjustment factors (which vary) to account for the lower dose fractions absorbed by target tissues at lower, more environmentally-relevant water concentrations and doses as compared to the dose fraction absorbed at the water concentration and dose (1.1 mg Cr/kg-day at 14 mg SDD/L) corresponding to the POD for the draft SFo ($BMDL_{10}$ values of 1–1.1 mg/kg-day). For example, the 4-fold difference between the dose fraction absorbed at the MCL versus that at the water concentration corresponding to the draft SFo POD (see Table 9) indicates that the draft SFo over-predicts cancer risk by around four times even at the MCL (0.1 mg/L), (note that when the mouse dose at the MCL is converted to a human equivalent dose, the human dose is that expected for humans at the MCL) which is over 500 times higher than typical drinking water levels (e.g., 35-city GM of 0.00018 mg/L reported in EWG, 2010). Using this factor of 4 to adjust the draft SFo for the estimation of risk at the approximate human dose (2.9E−03 mg/kg-day) associated with the MCL results in an excess risk of about 3.6E−04. However, even the one-third of the MCL evaluated in this study is almost 200 times higher than typical drinking water concentrations (e.g., GM of 0.00018 mg/L), and the 6-fold difference between the dose fraction absorbed at one-third of the MCL versus that at the draft SFo POD (see Table 9) indicates that the draft SFo over-predicts cancer risk by around six times even at this high, atypical drinking water concentration (0.033 mg/L). In fact, the highest drinking water concentration (0.0129 mg/L) reported in EWG (2010) is only about one-eighth of the MCL. Perhaps even more pertinent to the propensity of the draft SFo to over-estimate environmental risk, estimates of the much lower dose fractions that may be absorbed at environmentally-relevant concentrations (0.00018–0.0129 mg/L) suggest that risk over-estimation by the draft SFo for drinking water concentrations that humans are likely to be exposed to may very well span orders of magnitude (tens to perhaps thousands). Furthermore, based on alternative MOAs, the carcinogenic risk at low (i.e., environmental) doses could be as low as zero (Thompson et al., 2013).

Considering that risk over-estimation by the draft SFo for environmentally-relevant drinking water concentrations is likely to be at least an order of magnitude and may span multiple orders of magnitude, these analyses suggest the draft SFo be divided by a dose-specific adjustment factor of at least an order of magnitude (adjusted SFo of $\leqslant$0.05 per mg/kg-day) to be less over-predictive of risk at human-relevant doses (e.g., 0.0129 mg/L). Use of an example adjusted SFo to conservatively estimate risk at the approximate human dose (3.7E−04 mg/kg-day) corresponding to the highest reported CrVI tap water concentration (0.0129 mg/L) from EWG (2010) results in a high-end excess risk estimate no greater than 1.9E−05. This CrVI drinking water risk is well within USEPA's acceptable risk range (1E−06–1E−04).

Potential limitations of this study include the assumption that 90-day tissue concentration data (Kirman et al., 2012) are representative of those for longer-term exposure (NTP, 2008) in a relative (not absolute) manner. That is, that the relative proportions of the dose fractions absorbed at various doses do not change significantly with longer exposure. The lack of target tissue data at truly environmentally-relevant drinking water concentrations and doses and the use of modeling (i.e., BMD, proportionality calculations) to account for this is another limitation and/or uncertainty associated with some analyses, although the information available supports the approaches utilized. Although the relative simplicity of the approach employed in the current study may be viewed as a limitation compared to the more elegant PBTK models that have been developed (Kirman et al., 2012, 2013), its straightforwardness and ease of understanding can also be viewed as strengths. An assumption inherent in adjusting an SFo in this manner based on dose fraction absorbed is that risk is proportional to target tissue dose. However, the assumption that target tissue dose is linearly related to risk is inherently part of the default linear low-dose extrapolation method commonly used in regulatory risk assessment and regarded as conservative (i.e., health protective, no threshold is assumed). On the other hand, although the present study assumes low-dose linearity of target tissue dose (not oral dose) and risk (i.e., a mutagenic MOA), this paper should not be viewed as an endorsement of it in the MOA debate, as this approach may not be the best supported low-dose extrapolation method for CrVI oral risk assessment (e.g., estimating risk at environmental doses) based on the available information relevant to the MOA for CrVI-induced oral carcinogenicity (e.g., Thompson et al., 2011a, 2013). Performing a weight-of-evidence on the most likely carcinogenic MOA, however, is beyond the scope of this paper.

Conflict of interest

Nothing to disclose.

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Research Paper

Characterization of the Human Upper Gastrointestinal Contents Under Conditions Simulating Bioavailability/Bioequivalence Studies

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Purpose. This study was conducted to compare the luminal composition of the upper gastrointestinal tract in the fasted and fed states in humans, with a view toward designing *in vitro* studies to explain/predict food effects on dosage form performance.
Methods. Twenty healthy human subjects received 250 mL water or 500 mL Ensure plus® (a complete nutrient drink) through a nasogastric tube and samples were aspirated from the gastric antrum or duodenum for a period up to 3.5 h, depending on location/fluid combination. Samples were analyzed for polyethylene glycol, pH, buffer capacity, osmolality, surface tension, pepsin, total carbohydrates, total protein content, and bile salts.
Results. Following Ensure plus® administration, gastric pH was elevated, buffer capacity ranged from 14 to 28 mmoL L^{-1} ΔpH^{-1} (*vs.* 7–18 mmol L^{-1} ΔpH^{-1}), contents were hyperosmolar, gastric pepsin levels doubled, and surface tension was 30% lower than after administration of water. Post- and preprandial duodenal pH values were initially similar, but slowly decreased to 5.2 postprandially, whereas buffer capacity increased from 5.6 mmol L^{-1} ΔpH^{-1} (fasted) to 18–30 mmol L^{-1} ΔpH^{-1} ($p < 0.05$). Postprandial surface tension in the duodenum decreased by >30%, bile salt levels were two to four times higher, luminal contents were hyperosmotic, and the presence of peptides and sugars was confirmed.
Conclusions. This work shows that, in addition to already well characterized parameters (e.g., pH, and bile salt levels), significant differences in buffer capacity, surface tension, osmolality, and food components are observed pre-/postprandially. These differences should be reflected in test media to predict food effects on intralumenal performance of dosage forms.

KEY WORDS: Ensure plus®; fasted state; fed state; human gastric fluid; human intestinal fluid.

INTRODUCTION

The *in vivo* performance of oral dosage forms is an important issue when a new chemical entity is to be administered orally for first time in humans, when scale-up and postapproval changes to the dosage form are made, and when a generic formulation is to be evaluated for marketing authorization. To date, relevant bioavailability (BA)/bioequivalence (BE) information is obtained mostly with studies performed in healthy humans, making the procedure time-consuming and costly. During the last decade, the Biopharmaceutics Classification System (BCS) has introduced the possibility of obtaining a marketing approval for a generic formulation based on dissolution test results in certain cases (1), whereas the development of biorelevant media for the *in vitro* assessment of intralumenal fate of dosage forms has improved our ability to predict *in vivo* performance (2–4).

The initial compositions of the biorelevant media were based on existing intralumenal data (4–7). For various reasons, those data may not optimally reflect the *in vivo* situation during a standard BA/BE study. First, the buffer capacity, which is of primary importance for the dissolution characteristics of ionizable compounds, has not been well characterized in humans and the buffer capacity of biorelevant media had to be based on canine data (5). Second, in the fasted state, intragastric composition may be highly dependent on the volume of coadministered water and, although in BA or BE studies a standard volume of water is coadministered with the dosage form (8,9), characterization of human gastric environment has in many cases been performed without administration of water, or with unspecified volumes (10–15). Third, although the distribution of nutrients in the meals administered to characterize intralumenal conditions in the fed state was generally similar in previous relevant studies, meal energy content (which will affect gastric residence time) varied dramatically; in most previous studies total energy content was substantially lower [e.g., 158 kcal

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(10) or 300 kcal (16)] than the 800–1,000 kcal content of the FDA-recommended standard meal (17). Moreover, in studies where the meal composition was similar to the FDA meal, the intralumenal environment was only partly characterized (11,13,18). In addition to the buffer capacity, few or no data for osmolality and surface tension data in the fed state have been reported in the literature. Finally, in some studies, agents that induce the fed state rather than an actual meal have been administered to induce "fed state" conditions (19,20). This would minimize the intralumenal volume generated and, as a result, the intralumenal concentrations may be exaggerated compared to those generated during a BA/BE study.

In this study we characterized the upper gastrointestinal (GI) contents under conditions simulating BA/BE studies both in the fasted and in the fed states with a view toward designing media for the *in vitro* study of food effects on dosage form performance. In doing so, we gave emphasis to parameters that have not been fully characterized in the past and/or which are expected to vary with the composition of the administered meal. Fasted state conditions were simulated by administering 250 mL of water to fasted subjects, whereas fed state conditions were simulated by administration of 500 mL of Ensure plus® (21,22). It has previously been shown that Ensure plus® has a similar composition to that of the FDA meal that is commonly administered to study food effects in BA/BE studies (22).

MATERIALS AND METHODS

Phases of the Study

The study consisted of four phases. Each subject was administered a specific volume of fluid in the stomach and samples were aspirated either from the stomach or from a location lower than the sphincter of Oddi in the duodenum as follows:

Phase 1: Samples were aspirated from the antrum of stomach, after administration of 250 mL of water to the antrum through a nasogastric tube.

Phase 2: Samples were aspirated from the antrum of stomach, after administration of 500 mL of Ensure plus® (21,22) to the antrum through a nasogastric tube.

Phase 3: Samples were aspirated from the duodenum after administration of 250 mL of water to the antrum through a nasogastric tube.

Phase 4: Samples were aspirated from the duodenum after administration of 500 mL of Ensure plus® (21) to the antrum through a nasogastric tube.

Subjects

Twenty healthy nonsmokers (16 males and 4 females) with a mean age of 25 years (range 20–32 years) gave informed consent and participated in the study. One subject was 24% heavier than his ideal body weight [as determined from the Metropolitan Life Tables (23)]. Body weights of all other subjects deviated from the ideal weights by less than 10%. None of the participants had a history or any clinical evidence of gastrointestinal disease. The health status of each subject was confirmed by physical examination and screening of blood parameters for renal and hepatic functions.

The study was held in the Red Cross Hospital of Athens after receiving approval by the Scientific and the Executive Committee of the Hospital. The study followed the tenets of the Declarations of Helsinki promulgated in 1964.

From the 80 phases initially planned (20 subjects × 4 phases per subject), a total of 62 phases were successfully completed.

Four phases were incomplete for the following reasons:

– subject vomited during the aspiration period (two phases; one 45 min and another 33 min after administration of Ensure Plus®);

– movement of the tube toward the stomach (two phases; one 40 min and another 100 min after administration of Ensure Plus®).

Fourteen phases were not been performed for the following reasons:

– failure to position the duodenal lumen within a reasonable period (approximately 15 min) (7 phases);

– failure to aspirate samples from the duodenum partly due to creation of a vacuum in the duodenum (two phases);

– subject's decision to terminate his/her participation in the study (five phases).

Study Protocol

The study was performed on two separate experimental days in each subject. Alcohol and any over-the-counter medication were discontinued 3 days prior to and throughout each experimental day, whereas food intake was discontinued for at least 12 h prior to the start of each experimental day and water was restricted on the morning of the experimental day. At about 8 AM on the experimental day, the subject arrived at the clinic and, after a brief screening of his/her health status by a physician, the upper throat was sprayed with lidocaine.

Experimental Day A

The subject was intubated nasally using a sterile disposable tube (Levin #14). The tube is approximately 120 cm long with an external diameter of 4.9 mm. The tube was placed in the antrum of the stomach (under fluoroscopic guidance) and used for both manual administration of meals and manual aspiration of samples. Two hundred and fifty milliliters of mineral water containing 10 mg mL^{-1} PEG 4000 as a nonabsorbable marker were administered through the tube and ~20-mL samples were drawn and placed immediately on ice every 20 min for 60 min. Ninety minutes after water administration, 500 mL of Ensure Plus® containing 10 mg mL^{-1} PEG 4000 were administered to the antrum through the tube, and ~20-mL samples were drawn and placed immediately on ice every 30 min for 210 min. Immediately after each sample was taken, 20 mL of air was pumped into the sampling tube to clear the contents back into the lumen (total internal volume of the tube was estimated to be ~13 mL). After the last sample and before removing the tube/discharging the subject, the position of the tube was confirmed fluoroscopically.

Experimental Day B

The subject was intubated nasally using a sterile two lumen duodenal tube (model 455400 ch.15.0 Ruesch, Stuttgart, Germany). In contrast to some previous aspiration studies [e.g., (14)], no attempt was made to isolate the aspiration segment from the rest of the GI contents. The two lumen tubes were approximately 150 cm long with an external diameter of 4.7 mm and a metal tip at its distal end. A series of holes 27–36 cm proximal to the metal tip was used to access the antrum of the stomach. A further series of holes 0–10 cm proximally to the metal tip was used to aspirate samples from the duodenum (near the ligament of Treitz). Insertion of the tube was assisted by a hydrophilic guiding wire and its position was monitored fluoroscopically. After reaching its final position and removing the wire, 250 mL of mineral water containing 10 mg mL^{-1} PEG 4000 as a nonabsorbable marker were administered using 60-mL (capacity) syringes to the antrum. Thirty minutes after administration of water a ~20-mL sample from the duodenum was aspirated over ice. One hour after administration of water, 500 mL of Ensure Plus® containing 10 mg mL^{-1} PEG 4000 were administered to the antrum using 60-mL syringes over a period of 8–10 min. Samples of up to 20 mL were aspirated over ice from the duodenum every 30 min for 210 min after completion of administration of Ensure plus®. Immediately after each sample was taken, 20 mL of air was pumped into the tube to clear its contents back into the lumen (total internal volume of this sampling tube was estimated to be ~18 mL). At the end of the experimental day and before removing the tube/discharging the subject, the final position of the tube was confirmed fluoroscopically.

Handling and Analysis of Samples

Each aspirated sample was immediately divided into several subsamples and each subsample was used for measuring just one parameter.

pH and buffer capacity measurements were performed on the first subsample immediately upon aspiration. pH values were measured by a pH electrode (ER350B, Metrohm, Herisau, Switzerland). Because of subsample volume restrictions, buffer capacities were measured in just one pH direction, by dropwise addition of either NaOH (samples from fasted stomach) or HCl (samples from fed stomach, fasted duodenum, and fed duodenum). It is worth mentioning that titrating FaSSIF or FeSSIF with HCl has indeed proven to be more appropriate than titrating with NaOH (*24*). Buffer capacity was calculated according to the following definition: the sample has a buffer capacity value of 1 when one equivalent of strong acid or alkali is required to change the pH value of 1 L by one pH unit (25,26).

Effect of Sample Handling on pH Results. The pH of some subsamples was also measured after maintaining the sample at room temperature without stirring for up to 20 min, to determine whether any drift in the value with time/exposure to open air occurs.

Protein Content: Immediately upon aspiration and before storage at −70°C, gastric subsamples in which total protein content was to be measured were titrated to pH 1 to inhibit proteolytic activity of pepsin (27). Similarly, in duodenal aspirates phenylmethylsulfonyl fluoride (PMSF) was added to inhibit trypsin activity by achieving an end concentration of 1 mM (28). Total protein content was determined using a commercially available kit (BCA, Protein Assay Reagent Kit; Pierce, Rockford, IL, USA) and albumin as a standard. The quantification limit was calculated (29) every analytical day and it was always less than 0.300 mg mL^{-1}.

Pepsin Activity: Immediately upon collection and before storage at −70°C, gastric subsamples in which pepsin activity was to be measured were titrated to pH 6 (27). Pepsin activity was measured by a modification of the method described by Anson (30), and quantification was based on hog pepsin as a standard. The quantification limit was calculated (29) to be 0.010 mg mL^{-1}.

All the remaining subsamples were stored at −70°C immediately after collection. Surface tension was measured using the DeNouy ring method (Sigma70, KSV Instruments, Monroe, CT, USA). Osmolality was measured by using the freezing point depression technique (semimicro osmometer Typ Dig L; Knauer, Berlin, Germany). Total 3α-hydroxy bile acid levels were determined using a commercially available kit (Enzabile; Nycomed, Lidingö, Sweden) and the quantification limit (29) was 500 μM. This kit should only be used for assaying 3α-hydroxy bile salts in simple aqueous samples and/or nonprotein based media after appropriate dilution of the sample with equine serum (which does not contain any bile salts) [e.g., (31)]. PEG 4000 was determined by the method described by Malawer and Powell (32) and modified by Buxton *et al.* (33). The quantification limit (29) of the polyethylene glycol (PEG) assay method was 3.33 mg mL^{-1}. Total carbohydrate content was determined by a modification of the method described by Galanos and Kapoulas (34), using glucose as a standard. The quantification limit (29) was 0.800 mg mL^{-1}.

The physicochemical characteristics of the administered meals, determined using the analytical techniques described above and information from the manufacturers, are presented in Table I.

Data Analysis

Data are presented as box plots showing the median value, the 10th, 25th, 75th, and 90th percentiles, and the

Table I. Physicochemical Characteristics of Administered Liquid Meals

	Water containing 10 mg mL^{-1} PEG	Ensure Plus® containing 10 mg mL^{-1} PEG
Volume (mL)	250	500
Calories (kcal)	0	750
Osmolality (mOsm kg^{-1})	16	610
pH	7.8	6.6
Buffer capacity (mmol L^{-1} ΔpH^{-1})	1	24
Surface tension (mN m^{-1})	62.0	42.4
Total proteins (mg mL^{-1})	–	62
Carbohydrates (mg mL^{-1})	–	202
Fat (mg mL^{-1})	–	49.2

outlier data points, with triangles indicating the mean value. The number of subjects that contributed to a specific box plot is indicated in parentheses above/below each box. Data from a minimum of four subjects was used as the basis for constructing a box plot. Only the data exceeding the quantification limit (LOQ) have been included in the box plots. Data biased to higher values because the number of samples less than LOQ was equal to or greater than the number of samples with greater than LOQ are clearly designated in the text. For each parameter, differences between times were evaluated with one-way ANOVA or the Kruskal–Wallis test. When data did not vary with time, differences between pooled fasted data and pooled fed data were performed with the unpaired *t* test or the Mann–Whitney test. Decision on the use of a parametric or a distribution-free test was made on the basis of the normality and the equal variance tests. Comparisons of pH data were always made with distribution-free tests. All statistical comparisons were performed using Sigmastat 2.03 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Fasted Stomach

Data measured in the fasted stomach are presented in Fig. 1.

Polyethylene Glycol: Median PEG concentration 20 min after administration of water was only 40% of the input value (4.0 mg mL^{-1}). Because the number of samples with PEG concentrations <LOQ was the same as the number of samples having values >LOQ (nine, Fig. 1), data are biased to higher values. Therefore, at 20 min, at least 60% of the contents must have consisted of secretions. Because resting volumes are of the order of 25 mL (35), this rather substantial dilution is attributed to secretions by the gastric mucosa, incoming saliva, and, possibly, incoming nasal secretions generated by the presence of the tube into the nostrils and/or the pharynx (36). Due to sample volume limitations, data at later time points were not collected. The dilution of contents

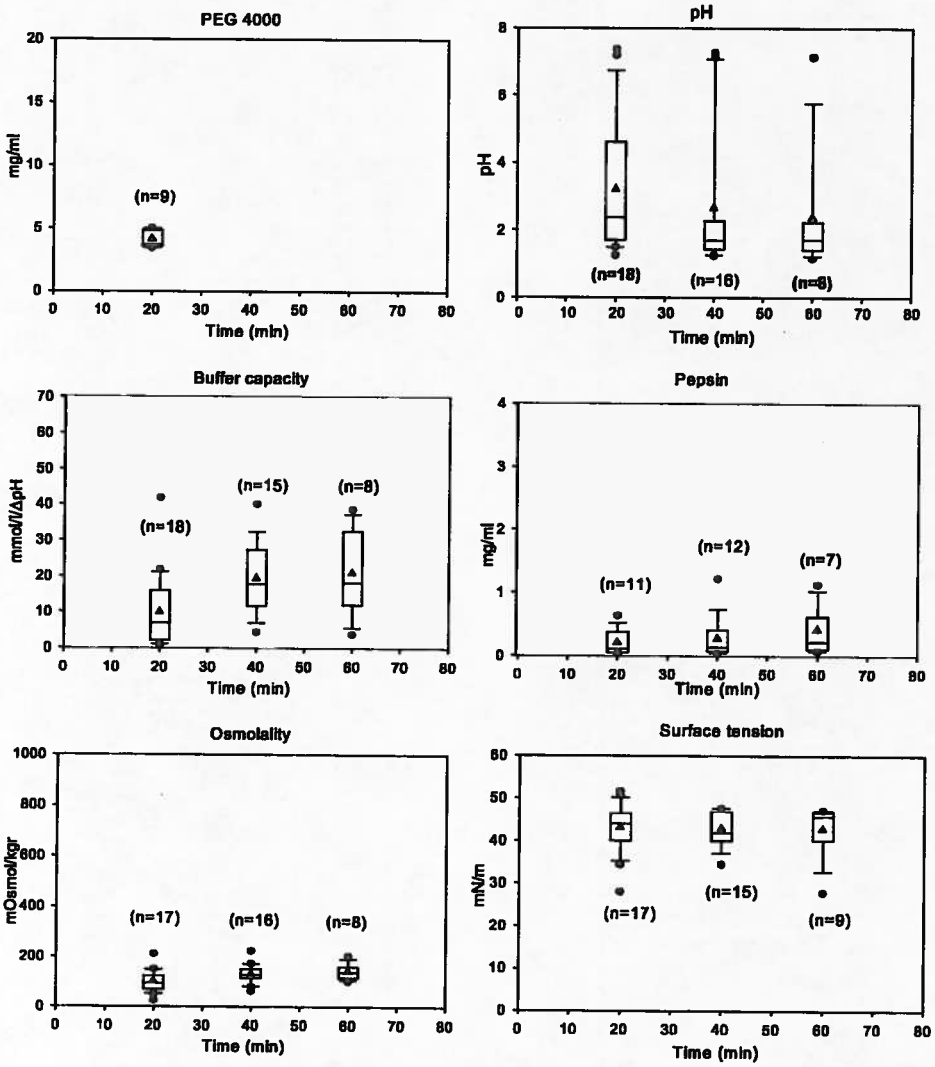

Fig. 1. Box plots for the samples aspirated from the antrum of fasted healthy subjects after administration of 250 mL water containing 10 mg mL^{-1} PEG 4000 into the antrum.

within the 20-min period after administration of water had an impact on pH, buffer capacity, pepsin levels, and osmolality.

pH: Intersubject variation was high (range of individual pH values was 1.23–7.36). Extreme high pH values may in some cases reflect an underlying hypochlorhydria [two subjects in our study consistently showed (at all sampling times) pH values close to neutral], but in most cases they probably reflect the dilution of gastric contents with saliva and/or nasal secretions [the baseline pH of saliva ranges from 5.45 to 6.06 and upon stimulation, the pH rises by about two pH units to a maximum of 7.8 (37)]. Median pH value was 2.4 twenty minutes after administration of water and stabilized to 1.7 at later time points. However, the decline over time did not achieve statistical significance ($p = 0.223$). pH values of 1.7 at late time points are in agreement with the generally accepted value for fasting gastric pH, which is usually measured to be

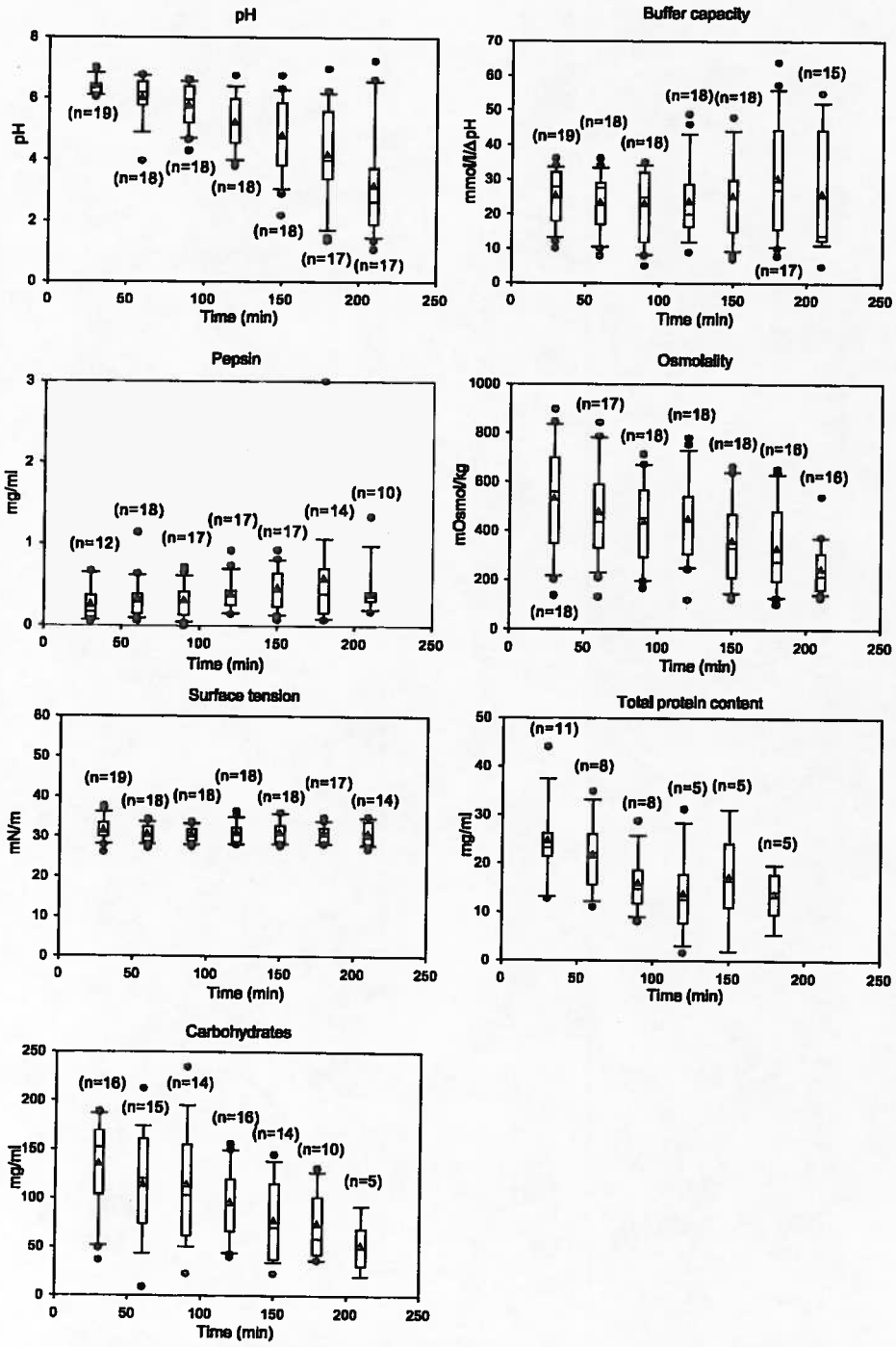

Fig. 2. Box plots for the samples aspirated from the antrum of fasted healthy subjects after administration of 500 mL Ensure Plus® containing 10 mg mL^{-1} PEG 4000 into the antrum.

about 2 or slightly lower (12–17). The pH of fasting gastric aspirates remained unchanged when the samples were maintained at room temperature for up to 20 min.

Buffer Capacity: The median value for buffer capacity 20 min after administration of water was 7 mmol L^{-1} ΔpH^{-1} and increased to about 18 mmol L^{-1} ΔpH^{-1} at later time points ($p < 0.001$).

Pepsin: Median values ranged from 0.11 mg mL^{-1} at 20 min to 0.22 mg mL^{-1} at 60 min (NS, $p > 0.05$). In the literature, values reported for pepsin concentration are higher. Schmidt *et al.* (19) report a value of 0.87 mg mL^{-1} (determined by hydrolysis of hemoglobin at pH 1.7 and by using hog pepsin as standard) and Lambert *et al.* (38) reported values of 0.83–1.27 mg mL^{-1} (determined with an analytical technique similar to that used in this study). An important methodological difference between the present and the previous studies is that no water seems to have been administered prior to collecting aspirates in the previous studies (19,38).

Osmolality: Although gastric contents were clearly hyposmotic, osmolality was lower at early time points (98 mOsm kg^{-1} at 20 min) and plateaued to about 140 mOsm kg^{-1} at later times ($p = 0.026$). These values are consistent with those reported earlier by Gisolfi *et al.* (39) (mean of 29 mOsm kg^{-1} over an 85-min exercise period during which 1,850 mL water was concurrently administered), by Lindahl *et al.* (14) (191 mOsm kg^{-1}, without prior water administration), and by Davenport (37) (171–276 mOsm kg^{-1}, no indication of whether water was preadministered).

Surface Tension: Surface tension was practically unaffected by water administration, with median values ranging from 41.9 to 45.7 mN m^{-1} during the first hour after the administration of water. These values are similar to previously reported results (40,41). Some investigators have attributed the low surface tension of gastric contents (pure water has a surface tension of 72 mN m^{-1}) to a reflux of duodenal contents (42,43). However, others have shown that this cannot be the reason in all subjects, as in many of them the bile salt levels in gastric aspirates are below the limits of detection (41,44–46). It is interesting to note that pepsin alone (at physiological relevant concentrations) is able to decrease the surface tension of water to about 57 mN m^{-1} (4).

Bile Acids: Bile acids, if any, were present at concentrations below the quantification limit of analytical method used in this study (i.e., less than 500 μM). However, bile salts at concentrations up to 1 mM (refluxed from the duodenum) have been quantified by other research groups in the fasted stomach (14,41,43,47). It is worth mentioning that in some of the earlier studies, samples were aspirated from the resting gastric contents rather than during gastric emptying of water from the stomach. From convectional considerations, one might reasonably expect that duodenal reflux would be more pronounced during resting conditions than during active gastric emptying of a liquid.

Fed Stomach

Data for the fed stomach are presented in Fig. 2. In agreement with literature data (33), the analytical method employed in our study for measuring PEG levels was not reliable in the presence of high nutrient concentrations. Therefore, an accurate picture of dilution or concentration of gastric contents over time in the fed state was not possible.

pH: Thirty minutes post-Ensure Plus® administration, the median gastric pH was 6.4 and intersubject variability was low. This value is close to the pH value of Ensure Plus® (6.6). Although intersubject variability increased with time, median pH values gradually decreased ($p < 0.001$) to reach 2.7 at 210 min, indicating that the meal effects on intragastric pH were still apparent 3 h and 30 min after the meal was given. The time required to restore the fasting pH levels depends mainly on the composition and the quantity of the meal, whereas the input pH value seems to be of secondary importance. For example, the time for gastric pH to return to fasting levels after administration of 580 mL of a pH 5.6 meal (651 mOsm, 1,000 kcal) was about 2 h (13), whereas after administration of 400 mL of a pH 6 meal (540 mOsm, 458 kcal) it was about 1 h (11). pH of gastric aspirates drifted in both directions by 5–25% within 15 min when the samples were kept at room temperature.

Buffer Capacity: As with pH, variability increased with time. Unlike pH, no trend in the median value was apparent. During the 30- to 210-min sampling period, median values of buffer capacity ranged from 14 to 28 mmol L^{-1} ΔpH^{-1}, close to input value (Table I). These values are significantly higher than the values measured 20 min after administration of water ($p < 0.001$), but are not different from values measured at times longer then 20 min after the administration of water. Higher total buffer content of gastric contents in the fed compared to the fasting state has also been reported by others; when 10 mL of homogenized meal (500 mL, 546 kcal, ~50% from lipids) was incubated with 20 mL of fresh gastric juice, a 33% increase in buffer capacity in 2 h was generated (48).

Pepsin: Both median values and intersubject variability remained fairly constant with time. During the 30- to 210-min sampling period, pepsin levels ranged from 0.26 to 0.58 mg mL^{-1}. These values are significantly different ($p = 0.006$) and up to twice as high as those measured in fasted state (Fig. 1). However, they are lower than the values found in the study of Lambert *et al.* (38), where pepsin levels in gastric aspirates after intravenous administration of insulin or betazole (histalog) were reported to be 0.56–1.72 mg mL^{-1} and also lower than the values found in the study of Schmidt *et al.* (19), where values of 1.25 and 1.68 mg mL^{-1} were reported after stimulation with histamine or with insulin, respectively. Again, these differences can be attributed to differences in the study protocols; in the earlier studies, which recruited subjects hospitalized for various disorders, induction of the fed state was performed pharmacologically rather than by administration of a meal, leading to substantially lower intragastric volumes and thus, higher pepsin concentration.

Osmolality: Both the median value and intersubject variability decreased with time after ingestion of the meal. The median value 30 min after the administration of the Ensure Plus® was 559 mOsm kg^{-1}, whereas at 210 min it decreased to 217 mOsm kg^{-1} ($p = 0.001$). Mertz and Poppe (20) reported a range of 262–306 mOsm kg^{-1} for osmolality after intravenous infusion of betazole (an analog of histamine); therefore gastric secretions under fed simulating conditions are isoosmotic or only slightly hyperosmotic and the high osmolality of fed aspirates in this study can be attributed to the hyperosmolarity of the administered meal.

Surface Tension: Surface tension values showed remarkable reproducibility and during the entire aspiration period median values ranged from 30 to 31 mN m^{-1}, i.e., they were 30% lower than in the fasting state ($p < 0.001$).

Bile Salts: Only one sample had a bile content above the quantification limit (i.e., higher than 500 μM). In the literature trace levels of bile salts have been reported to be present in the fed stomach [mean value = 60 μM (43)]. Because of the high limit of quantification of our method, it is not surprising that no bile salts could be detected in the stomach either fasted or fed and it is not possible from our results to say whether bile salts are refluxed or not.

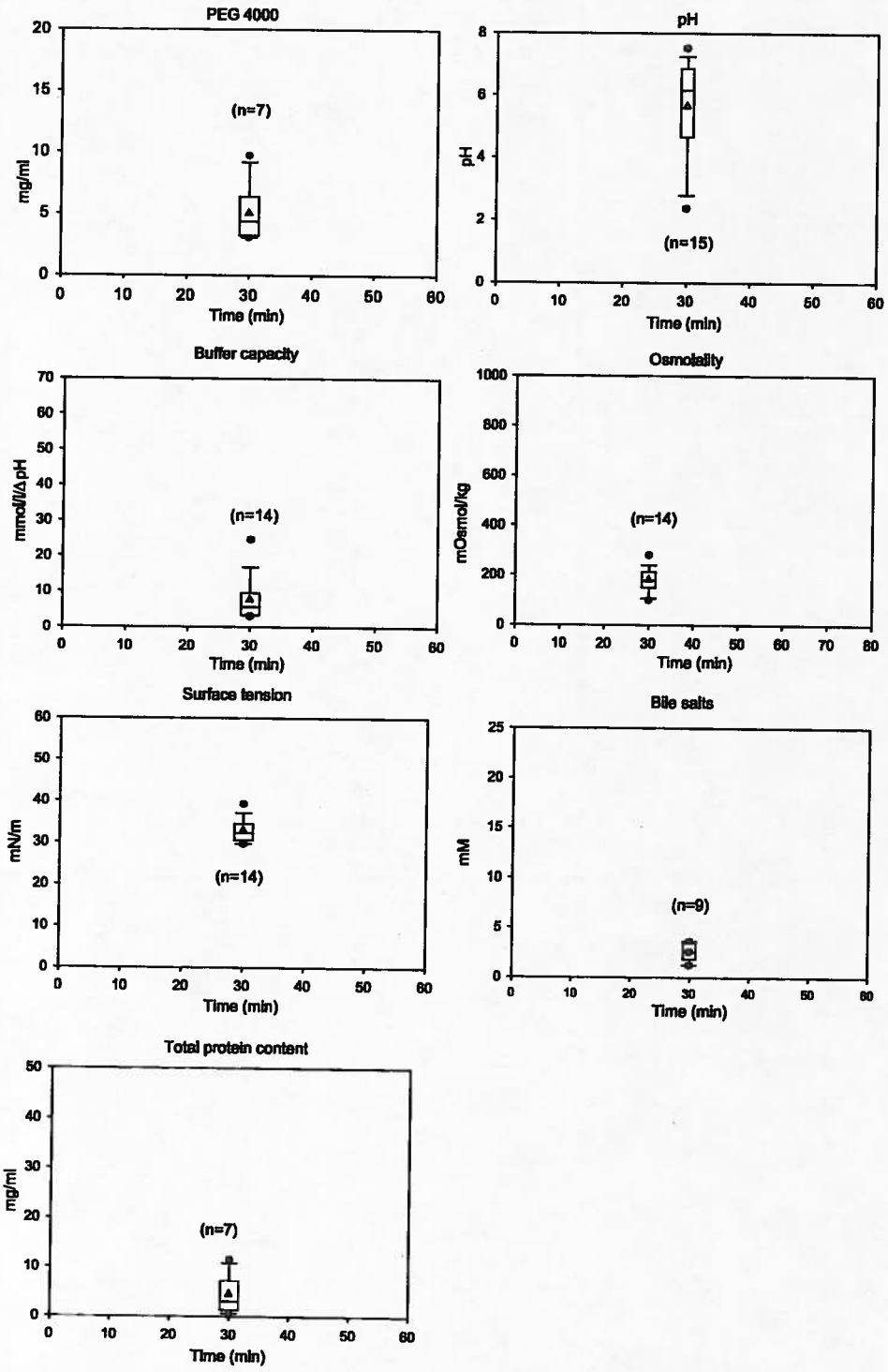

Fig. 3. Box plots for the samples aspirated from the distal duodenum of fasted healthy subjects after administration of 250 mL water containing 10 mg mL^{-1} PEG 4000 into the antrum.

Total Protein and Carbohydrate Content: The median values for total protein and total carbohydrate content decreased gradually from 23.3 and 152.1 mg mL^{-1}, respectively, at 30 min to 11.2 and 49.1 mg mL^{-1}, respectively, at 210 min after the meal's administration. The substantial presence of nutrients 210 min after administration of the meal is in accordance with the higher than baseline pH level discussed earlier. However, it should be noted that, in Fig. 2, total protein data are biased to higher values because the total number of samples with values <LOQ (sixty one) was higher

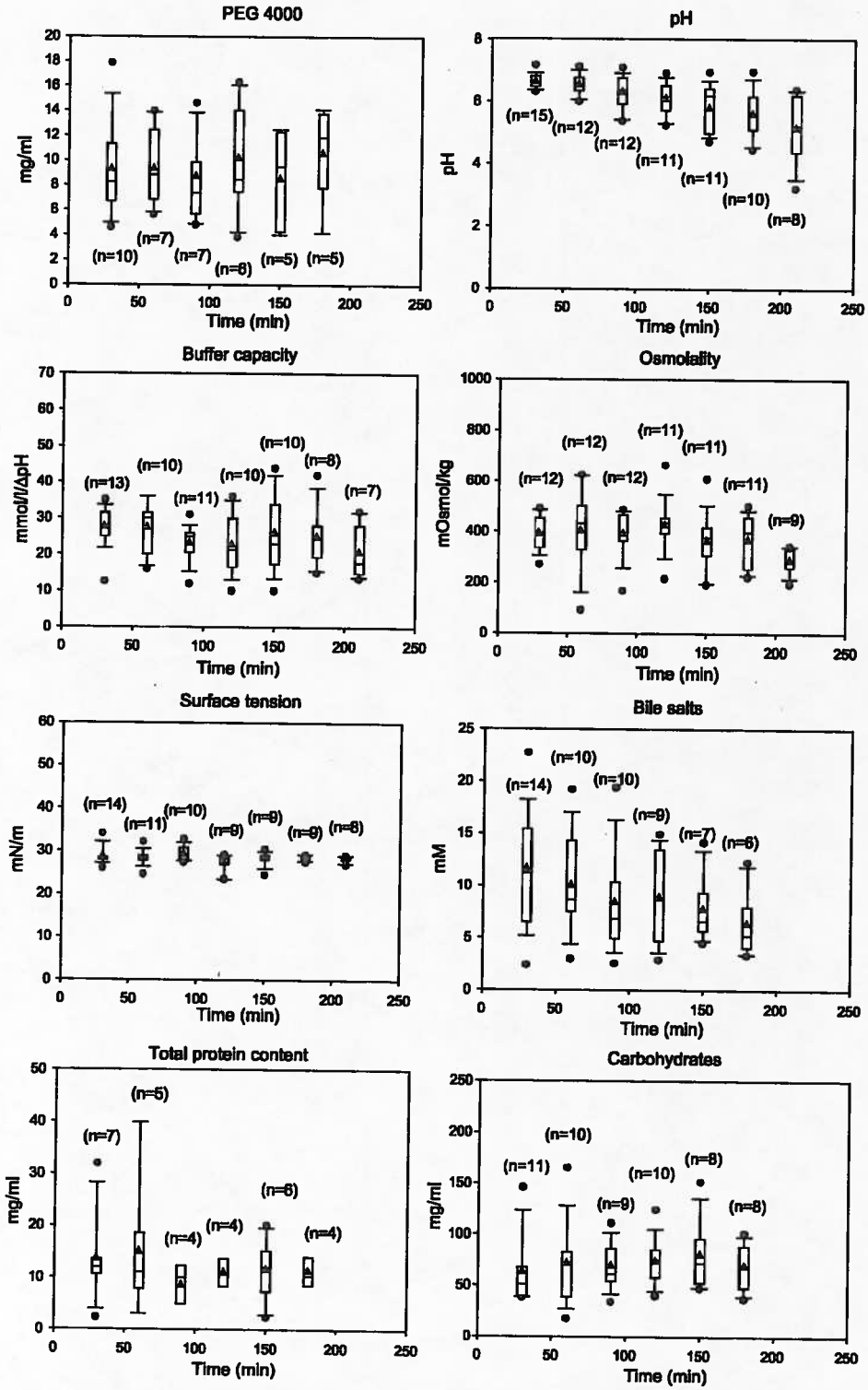

Fig. 4. Box plots for the samples aspirated from the distal duodenum of fasted healthy subjects after administration of 500 mL Ensure Plus® containing 10 mg mL^{-1} PEG 4000 into the antrum.

than the total number of samples with total protein values >LOQ (forty five, Fig. 2).

Fasted Duodenum

Data obtained from duodenal aspirates after administration of water are presented in Fig. 3.

Polyethylene glycol: Median PEG value was 4.3 mg mL^{-1}, similar to the gastric value 20 min after administration of water. These results provide an indication that any water absorption across the duodenal wall is balanced by the baseline bile and pancreatic secretions. However, it should be noted that, in Fig. 3, PEG data are biased to higher values because the total number of samples with values <LOQ (eight) was higher than the total number of samples with total protein values >LOQ (seven, Fig. 3).

pH: As in the fasting stomach, pH values were highly variable. However, the variability observed in the fasted duodenum may be related to reasons other than those speculated for the fasting stomach. According to Woodtli and Owyang (49), intersubject variations of the pH are related to the different phases of interdigestive motility. Moreover, in our study it was confirmed that the two subjects with the lowest intraduodenal pH values were those for which the tube was slightly moved upwards during the experimentation period and, therefore, aspirated sample corresponded to the descending part of the duodenum (i.e., close to or even proximal to the sphincter of Oddi). At the other extreme end, the subject with a slightly alkaline pH in the duodenum was the one that showed almost neutral gastric pH in the fasting state. The median pH value was 6.2. This is in agreement with median fasting duodenal pH values reported in the literature, which vary from 5.95 to 6.72 (10–13,15,50,51). pH of duodenal aspirates drifted to higher values, increasing by up to 6% within 20 min at room temperature. This could be related to a slow transformation of bicarbonates to carbon dioxide under zero-convection conditions (52).

Buffer Capacity: Median buffer capacity was 5.6 mmol L^{-1} ΔpH^{-1}, i.e., much lower than the median gastric value. To the best of our knowledge, there is only one relevant study in which the concentration of bicarbonates immediately next to the duodenal bulb was (albeit indirectly) measured. By measuring pH and partial pressure of carbon dioxide (53), the concentration was found to be about 6.7 mM.

Osmolality: Contents were hypoosmotic (median value = 178 mOsm kg^{-1}). Data are in accordance with those reported by Gisolfi *et al.* (39) (142 mOsm kg^{-1} in intestinal fluids aspirated 25 cm from pylorus) and, as would be expected, lower than the value reported by Lindahl *et al.* (14) for jejunal aspirates (271 mOsm kg^{-1}).

Surface Tension: As in the fasted stomach, this parameter showed the least variability. However, the median value (32.3 mN m^{-1}) was much lower than the gastric value, presumably as a result of the higher level of surface active agents such as bile salts and enzymes.

Bile Salts: The median value was 2.6 mM, similar to the value reported by Lindahl *et al.* (14) for the concentration of bile salts in jejunum during fasted state conditions (average 2.9 mM), but lower than the values found by other investigators for fasted duodenal contents [4.3–6.4 mM (5)].

Total Protein Content: The median value was 3.1 mg mL^{-1} and corresponds to enzymes arriving into duodenum from the stomach (e.g., pepsin, data not shown), the pancreas, and the bile duct [10 g of protein are secreted by the liver into the bile every day in a 70-kg man (54)]. However, this value probably overestimates the actual average intralumenal total protein content because the number of samples with values >LOQ (seven, Fig. 3) was only slightly higher than the number samples with total protein values <LOQ (six). It should be noted that the average total protein content of fasted jejunum has been reported to be 2.1 mg mL^{-1} (14).

Fed Duodenum

Data for the characterization of the luminal contents in the fed duodenum are presented in Fig. 4.

Polyethylene Glycol: Although median values were close to input PEG concentrations, data were extremely variable, suggesting that in some cases there was significant water absorption whereas in others there was significant water secretion. Postprandial values were on the average significantly higher than those 20 min after water administration ($p = 0.006$).

pH: Data were less variable than in the fasting state. The median duodenal pH 30 min after meal administration was 6.6, somewhat higher than the fasting state value, but it fell ($p < 0.001$) slowly to 5.2 at 210 min after the administration of Ensure Plus®. Although the pH decrease with time in the fed upper small intestine is known (10,11), earlier data had suggested that it occurs faster than in the present study and that perhaps the return to the higher pH levels of the fasted duodenal lumen was also faster (11). In the latter study, the energy content of the meal was 458 kcal, with 40% of calories coming from carbohydrates, 20% from proteins, and 40% from fats (11). The meal administered in the present study had similar percentage of calories coming from proteins (the major buffering species among nutrients), but contained much higher total energy content (Table I). Therefore, the buffer capacity is expected to be higher in this study and this could possibly account for the different timescale of progression of the pH value. The pH of duodenal aspirates drifted slightly by up to 3% to lower values within 10 min of storage at room temperature. This is speculated to be related to the creation of digestion products with acidic properties (e.g., digestion of triglycerides).

Buffer Capacity: Median values were between 18 and 30 mmol L^{-1} ΔpH^{-1} without showing a specific trend over time. These values are significantly higher than those measured after water administration ($p < 0.001$). Based on medians, the picture is similar to the corresponding gastric data in the fed state (Fig. 2). It is interesting to note that the extremes in buffer capacity results (high and low) corresponded with the extreme PEG values, i.e., with extremes in net water flux behavior. However, variability in net water flux did not impact the intraduodenal pH (as discussed above), presumably because intraduodenal pH values, the pH of the meal, and the pH of secretions are all close to neutral. Literature data on buffer capacity in the fed duodenum are very limited. Rune (55) measured the pH and the partial

pressure of carbon dioxide in samples aspirated 10 cm lower than the pylorus 3 and 3.75 h after administration of a meal (393 kcal with 39% fats and 51% carbohydrates); estimated bicarbonate concentrations were 10 and 23 mEq L^{-1} (55).

Osmolality: As with both the PEG and the buffer capacity data, osmolality data showed increased variability. The increased variability may be related to the extremes in net water flux observed in some cases and would support the diverse clinical data with regard to the effects of input osmolality on intralumenal water absorption and secretion (39,56–60) that have been reported in the literature. Based on median values, duodenal contents were hyperosmotic over most of the aspiration period but achieved isoosmolality (287 mOsm kg^{-1}) first at 210 min. Values were significantly higher than those after water administration ($p < 0.001$). Ensure plus® contains a disaccharide (25% of total carbohydrate is sucrose), and, like the FDA meal, complex carbohydrates (34% of total carbohydrate is maltodextrin). Gradual hydrolysis of carbohydrates and, perhaps, increased intestinal residence prior to their absorption [41% of total carbohydrates is corn syrup that contains mainly fructose; fructose is absorbed three to six times slower than glucose from the gut (36)] generate higher luminal osmolality (54).

Surface Tension: Surface tension, as in all previous phases, showed the least variability. Medians were very low and ranged between 28.1 and 28.8 mN m^{-1}. Values were significantly different from those measured after water administration ($p < 0.001$).

Bile Salts: Data showed higher variability in the fed state, but results tended to decrease and become more consistent with time. Extreme low values were associated with sampling from the upper-middle duodenum, i.e., close to or even proximal to the sphincter of Oddi. Medians dropped from 11.2 to 5.2 mM at 180 min postdosing. Armand *et al.* (61) reported mean values of 6.7–13.4 mM up to 4 h after a 960-kcal meal (67.5% of calories were from lipids). Fausa (62) reported a mean concentration of 14.5 mM for bile salts at 30 min after administration of the meal (300 mL) and 5.2 mM between 30 and 60 min after administration of the meal.

Total Protein and Carbohydrate Content: Total protein content and total carbohydrate content were variable but much lower than input values over the entire aspiration period. However, data for total protein content are probably biased to higher values because the total number of samples with values <LOQ (twenty nine) was only slightly less than the number of samples with values >LOQ (thirty, Fig. 4). The high total protein content even 180 min after administration of the meal, significantly different than the content measured after water administration ($p = 0.005$), can be attributed partly to the increased presence of enzymes and partly to the presence of proteins in Ensure plus®. Caseinates, as well as other phosphopeptides that are present both in Ensure plus® and in the meal administered in BA/BE studies, are known to be relatively resistant to enzymatic digestion, and their digestibility may be affected by the presence of starch (54). With regard to carbohydrates, significant amounts were still present 180 min after administration of the meal. Although some carbohydrates may be contributed by the bile (37,53), a part of the carbohydrates measured would have been contributed by the maltodextrins [i.e., degradation products of starch; 2–20% of dietary starch and, perhaps, fructose escape absorption in the small bowel (64–66)]. It is worth mentioning that the FDA meal, which is often administered in BA/BE studies, also contains fructose (in orange juice) and starch.

CONCLUSION

The foregoing results confirm that there are very substantial differences in well-characterized parameters such as pH and bile salt concentrations between the fasted and fed states. Furthermore, substantial differences were established in less well-characterized, but pharmaceutically important, parameters such as buffer capacity, osmolality, and volume of luminal contents. A key difference between this study and previous studies was the attempt to simulate usual dosing conditions in a bioavailability/bioequivalence study. For experimental reasons (potential for clogging of aspiration tubes), it was not possible to aspirate after administration of the standard FDA meal. However, a fluid "total nutrition drink" with very similar carbohydrate/protein/fat ratios as well as most other physical chemical properties to the standard meal was substituted, making it possible to aspirate and still at least approximate the conditions usually adopted in bioavailability/bioequivalence studies. In addition, there have been very few studies published that have attempted to characterize luminal conditions in the duodenum after the administration of a meal. The data reported here suggest that, although the current biorelevant media better simulate the luminal environment much more nearly than standard compendial media, there is still some room for improvement. These results, coupled with a separate set of results characterizing the lipids in the GI tract in the fed state, will be used to design a "second generation" of biorelevant media. Potential uses of these media would be for characterization of solubility, dissolution, and permeability properties of drugs and dosage forms.

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Environmental Contamination and Toxicology

Embryo- and Fetotoxicity of Chromium in Pregestationally Exposed Mice

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Chromium, an essential element in the human body required for proper carbohydrate, protein, and fat metabolism, is reported to impair gestational development of offspring of workers chronically exposed to this metal in the work place. Workers in chromium based industries can be exposed to concentrations two orders of magnitude higher than the general population (Hemminki and Vainio 1984). Among the general population, residents living near chromate production sites may be exposed to high levels of chromium (VI) in air or to elevated levels (40 - 50,000 ppm) of chromium in effluents (Rumar 1987). Shmitova (1978,1980) reported afterbirth and puerperal hemorrhages in women industrially exposed to this metal and observed high chromium levels in blood and urine of pregnant women and in fetal and cord blood. Chromium readily passes the placental barrier and reaches the growing fetus (Tipton 1960; Pribluda 1963). Exposure of mice to chromium during various gestational periods resulted in embryo and fetotoxic effects (Junaid et al. 1995, 1996).

Pribluda (1963) reported that the chromium content of bones of pregnant rats decrease with the advancement of gestation. Such released chromium may reach the circulatory system and enter feto-placental tissue through the placental barrier. Therefore, it was thought worthwhile to ascertain the role of body chromium accumulated pregestationally on embryo and fetal development and its subsequent transfer to feto-placental sites.

MATERIALS AND METHODS

Sixty, 4-month old, Swiss albino, female mice (body weight 30 ± 5 gms) of proven fertility from the Industrial Toxicology Research Centre colony were divided into four groups of fifteen mice each. Group I was given drinking water and served as the control, while groups II, III, and IV were treated with 250, 500 and 750 ppm chromium (VI, as potassium dichromate), respectively, in drinking water for 20 days [time required for complete development of an ovarian follicle (Pederson 1970]. The selection of doses was based on our earlier study (Trivedi et al. 1989) and the fact that the average chromium intake of humans is approximately 200 ug/day in drinking water (NRC 1989). The animals were individually housed

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under standard animal house conditions (room temprature 20-22°C, relative humidity 50±5%) where a regular cycle of 12 hrs light: 12 hrs darkness was maintained and were provided with feed pellets (Lipton India Ltd.) and water ad libitum. The dams were observed daily for water intake and clinical signs of toxicity. After 20 days, females were mated with normal healthy, adult males and females were checked for pregnancy the next morning. The day that the vaginal plug was found was designated as '0'day of gestation. Mothers were weighed and kept individually in plastic cages. Ten pregnant females were randomly selected from each group, weighed, and sacrificed on the 19th day of gestation under ether anesthesia and caesarian sections performed. Blood from five animals from each group was withdrawn from the heart in heparinized vials and kept at -20°C for chromium estimation. One fetus plus placenta/litter was also kept at -20°C for chromium estimation. Both ovaries were removed and the number of corpora lutea was determined. Total implantations, the number of fetuses/litter, the number of live/dead fetuses, crown-rump length, the number of resorptions, and the weight of the fetuses and their respective placenta were recorded. Pre and post-implantation loss (%) was calculated as described by Palmer et al. (1978). Remaining fetuses were examined for gross external abnormalities and 1/3 of these fetuses were fixed in Bouin's fluid for examination of visceral abnormalities (Wilson 1965), while the others were fixed in 95% ethanol, eviscerated, and stained by the Alizarin red S method (Staples and Schnell 1964) for examination of skeletal deformities (Kelsey 1974).

Known amounts of maternal blood, placentae and the fetuses were digested in Nitric acid:Perchloric acid (6:1) mixture till a white residue remained at the bottom of the flask. The residue was dissolved in 5.0 ml of 0.1 N Nitric acid and read on DC Plasma Emission Spectrophotometer (Beckman Spectrospan V). Blank and spiked samples were also run and analyzed simultaneously (Trivedi et al. 1989). The embryo- and feto-toxicity data in Table 1 and chromium estimation data in Table 3 were analysed by one-way ANOVA followed by Student's 't' test while gross and skeletal abnormalities data in Table 2 were analysed by Fischer's Exact Test (Brunning and Kintz 1977).

RESULTS AND DISCUSSION

The treated females did not show any notable change in behaviour or external features. Mortality (3 females; 20%) was observed in group IV. Autopsy of these animals could not establish the cause of death. Daily chromium (VI) intake as calculated by water consumed: 1.9 ± 0.02, 3.56 ± 0.03, and 5.23 ± 0.07 mg Cr for groups II, III, and IV, respectively. Water consumption in the control group was 8.52 ± 0.21 ml/mouse/day. No significant change in the weight of the mothers during the treatment was observed. Gestational weight gain of mothers in groups II and III was not significantly different when compared to controls; group IV registered no weight gain during gestation.

We observed an absence of implantation in the uterine horns of

group IV mothers. While corpora lutea were present, their numbers were significantly reduced compared to the rest of the treatment groups.

Group III had a significant ($P<0.05$) increase in the number of resorptions (37%) when compared with the control group. Decrease in fetal weight (39%) and crown rump length (28%) and increase in placental weight (63%) as well as pre-(25%) and post-implantation (37%) loss was evident in group III compared to the control group. No significant difference in the number of corpora lutea was observed in group III compared to group II.

There was a significant ($P<0.05$) decrease in fetal weight (30%), placental weight (7%) and crown-rump length (17%) and an increase in post-implantation loss (18%) in group II compared to the control (Table 1). No dead fetuses were observed in any of the treated groups.

The fetuses of group III had higher ($P<0.05$) number of sub-dermal haemorrhagic patches and kinky and short tails. The number was markedly higher than for the control and group II animals (Table 2)

No major skeletal abnormalites was observed in any of the treated groups. Significantly reduced ossification in caudal, parietal and interparietal bones of the fetuses of group III was observed in treated mothers (Table 2). Soft tissue examination did not reveal any significant deformities in any of the treated groups.

Blood chromium was signifcantly higher in group IV compared to all other groups whereas that of groups II and III was elevated compared to controls. Placental chromium concentration increased in a dose-dependent manner in groups II and III compared to controls. Fetuses of mothers in group III had significantly higher chromium concentrations compared to fetuses of control and group II mothers (Table 3).

Chromium (VI) is reported to pass the placental barrier and accumulate in fetal tissues (Shmitova 1980). The presence of chromium (VI) in fetuses and infants has been reported in women working or living near the dichromate industries (Shmitova 1978). It was also noticed that women working in chromium-based industries for many years experienced abnormal menses, which was attributed to ovarian-hormonal impairment (Ross 1978). Tipton (1960) reported the transfer of chromium from the mother to the bones of the developing fetus in humans. In rats, the pregestationally retained chromium is reported to pass to the developing fetuses if exposure is stopped during gestation (Pribluda 1963).

Chromium speciation, concentration, and duration of exposure are important variables influencing tissue distribution. Gastrointestinal uptake of chromium is 2 - 10 % of the dose in both humans and laboratory animals. Shiraishi and Ichikawa (1972) reported that the bones and kidneys of rats contained the highest chromium concentration in comparison to other tissues monitored following oral administration of chromium (VI).

Table 1. Chromium-induced embryo- and feto-toxicity in mice treated during the pregestational period.

Parameters	Group I (Control)	Group II (250 ppm)	Group III (500 ppm)	Group IV (750 ppm)
Weight gain in mothers (g)	14.40 ± 1.01	13.43 ± 0.50	12.38 ± 0.49	1.7 ± 0.93
Number of corpora lutea/mice	7.9 ± 1.01	7.4 ± 0.50	7.3 ± 0.37	4.4 ± 0.50 abc*
Number of implantations/mice	7.7 ± 0.74	6.8 ± 0.41	5.4 ± 0.27 a*	0
Number of live fetuses/mice	7.7 ± 0.74	5.6 ± 0.50	3.4 ± 0.24 ab*	0
Number of resorptions/mice	0	1.20 ± 0.44	2.0 ± 0.31 a*	0
Pre-implantation loss (%)	2.77 ± 1.21	8.38 ± 3.53	24.79 ± 2.17 ab*	100 %
Post-implantation loss (%)	0	17.51 ± 2.22 a*	36.66 ± 4.94 ab*	0
Fetal weight (g)	1.59 ± 0.04	1.11 ± 0.04 a*	0.97 ± 0.03 ab*	0
Placental weight (g)	0.137 ± 0.003	0.128 ± 0.005a*	0.223 ± 0.005ab*	0
Crown-rump length (cm)	2.92 ± 0.07	2.41 ± 0.08 a*	2.09 ± 0.08 ab*	0

Value represents mean ± S.E. of 10 female mice in each group.

The significance of the difference among various groups was evaluated by applying one-way ANOVA followed by Student's 't' test (Brunning and Kintz 1977).

* Significance $p < 0.05$. Comparison between two groups: a -vs control; b -vs 250 ppm; c -vs 500 ppm

Table 2. Incidences of gross and skeletal abnormalities in the pups of dams treated with chromium during the pregestational period.

Parameters	Group I (Control)	Group II (250 ppm)	Group III (500 ppm)
Gross abnormalities			
Number of pups/litters observed	72/10	51/10	19/10
Drooping rist	0/10	0/10	6/4 (32)
Sub-dermal hemorrhagic patches	0	8/6 (16)	8/4 (42) a*
Kinky tail	0	0	8/6 (42) a*
Short tail	0	4/4 (9)	10/4 (53) a*
Skeletal abnormalities			
Number of pups/litter observed	48/10	34/10	19/10
Reduced parietal ossification	0	0	12/10 (63) a*
Reduced inter-parietal ossification	0	0	10/10 (53) a*
Reduced caudal ossification	6/4 (12)	18/8 (53) a*	18/10 (95) a*

Gross and skeletal abnormalites are represented as number of abnormal pups/litters observed.

The statistical significance was evaluated by Fisher's Exact test (Drunning and Kintz 1977).

Percentage in parentheses calculated by the total number of pups observed.

* Significance $p < 0.05$. Comparison between two groups: a-vs control.

Table 3. Chromium concentrations in different tissues of mice treated during the pregestational period

Tissue	GroupI (Control)	Group II (250 ppm)	Group III (500 ppm)	Group IV (750 ppm)
Blood (µg/mL)	0.03 ± 0.007	0.05 ± 0.006 a*	0.06 ± 0.008 a*	0.13 ± 0.007 abc*
Placenta (µg/g:f.w.)	0.09 ± 0.001	0.14 ± 0.008 a*	0.17 ± 0.002 ab*	No implantation
Fetus (µg/g:f.w.)	0.04 ± 0.008	0.07 ± 0.007	0.16 ± 0.013 ab*	No implantation

Values represent mean ± S.E of 5 mice in each group.

The significance of the difference among various groups was evaluated by applying one-way ANOVA followed by Student's 't' test (Brunning and Kintz 1977). * Significance $p < 0.05$.

Comparison between two groups: a -vs control; b -vs 250 ppm; c -vs 500 ppm. f.w. q fresh weight.

In the present study, the treated animals showed an increase in blood chromium concentration compared to controls, with the highest dose group (750 ppm) having the highest chromium concentrations. However, blood chromium concentrations of the 250 and 500 ppm dose group were not significantly different from one another. This may be attributed to the fact that chromium (VI) enters the red blood cells where reduction to chromium (III) and subsequent binding to hemoglobin takes place. Assimilation of chromium (VI) in excess of the amount that can be reduced and sequestered results in longer residence time of chromium (VI) in blood and, hence, greater exposure of body tissues (Saner 1980). Although we have not assessed the extent of chromium transfer from maternal tissues to fetal tissue in the present study, the results from previous studies suggest transfer of prestored chromium from maternal soft tissue and/or bones to the developing fetus (Fitzgerald et al. 1985).

We observed a dose-dependent rise in placental chromium concentration as compared to the fetus. This may be due to the placenta acting as a barrier to retard passage of chromium from the mother to the fetus to safeguard fetal development and growth. The highest close group in this study (750 ppm) did not show any implantation. However, the release of ovum. as evidenced by the presence of corpora lutea, was apparent, although highly reduced in number compared to the rest of the treated and control groups. This reduction in number of corpora lutea may possibly be due to direct accumulation of chromium in ovarian tissue (Langard 1982) or reduced hormone levels (Mattison et al. 1983).

Pre-implantation loss (100%) in the highest dose group may also be attributed to reduced hormone levels (Mattison et al. 1983) or impaired embryos, as reported earlier (Jacquet and Draye 1982). Chromium passed to the fetus could have resulted in reduced fetal ossification, influencing fetal development either through a direct effect on fetal tissue (Matsumoto et al. 1976) or impairment of placental physiology (Faulk 1981).

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Research Paper

Characterization of the Human Upper Gastrointestinal Contents Under Conditions Simulating Bioavailability/Bioequivalence Studies

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Purpose. This study was conducted to compare the luminal composition of the upper gastrointestinal tract in the fasted and fed states in humans, with a view toward designing *in vitro* studies to explain/predict food effects on dosage form performance.
Methods. Twenty healthy human subjects received 250 mL water or 500 mL Ensure plus® (a complete nutrient drink) through a nasogastric tube and samples were aspirated from the gastric antrum or duodenum for a period up to 3.5 h, depending on location/fluid combination. Samples were analyzed for polyethylene glycol, pH, buffer capacity, osmolality, surface tension, pepsin, total carbohydrates, total protein content, and bile salts.
Results. Following Ensure plus® administration, gastric pH was elevated, buffer capacity ranged from 14 to 28 mmoL L^{-1} ΔpH^{-1} (*vs.* 7–18 mmol L^{-1} ΔpH^{-1}), contents were hyperosmolar, gastric pepsin levels doubled, and surface tension was 30% lower than after administration of water. Post- and preprandial duodenal pH values were initially similar, but slowly decreased to 5.2 postprandially, whereas buffer capacity increased from 5.6 mmol L^{-1} ΔpH^{-1} (fasted) to 18–30 mmol L^{-1} ΔpH^{-1} ($p < 0.05$). Postprandial surface tension in the duodenum decreased by >30%, bile salt levels were two to four times higher, luminal contents were hyperosmotic, and the presence of peptides and sugars was confirmed.
Conclusions. This work shows that, in addition to already well characterized parameters (e.g., pH, and bile salt levels), significant differences in buffer capacity, surface tension, osmolality, and food components are observed pre-/postprandially. These differences should be reflected in test media to predict food effects on intralumenal performance of dosage forms.

KEY WORDS: Ensure plus®; fasted state; fed state; human gastric fluid; human intestinal fluid.

INTRODUCTION

The *in vivo* performance of oral dosage forms is an important issue when a new chemical entity is to be administered orally for first time in humans, when scale-up and postapproval changes to the dosage form are made, and when a generic formulation is to be evaluated for marketing authorization. To date, relevant bioavailability (BA)/bioequivalence (BE) information is obtained mostly with studies performed in healthy humans, making the procedure time-consuming and costly. During the last decade, the Biopharmaceutics Classification System (BCS) has introduced the possibility of obtaining a marketing approval for a generic formulation based on dissolution test results in certain cases (1), whereas the development of biorelevant media for the *in vitro* assessment of intralumenal fate of dosage forms has improved our ability to predict *in vivo* performance (2–4).

The initial compositions of the biorelevant media were based on existing intralumenal data (4–7). For various reasons, those data may not optimally reflect the *in vivo* situation during a standard BA/BE study. First, the buffer capacity, which is of primary importance for the dissolution characteristics of ionizable compounds, has not been well characterized in humans and the buffer capacity of biorelevant media had to be based on canine data (5). Second, in the fasted state, intragastric composition may be highly dependent on the volume of coadministered water and, although in BA or BE studies a standard volume of water is coadministered with the dosage form (8,9), characterization of human gastric environment has in many cases been performed without administration of water, or with unspecified volumes (10–15). Third, although the distribution of nutrients in the meals administered to characterize intralumenal conditions in the fed state was generally similar in previous relevant studies, meal energy content (which will affect gastric residence time) varied dramatically; in most previous studies total energy content was substantially lower [e.g., 158 kcal

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(10) or 300 kcal (16)] than the 800–1,000 kcal content of the FDA-recommended standard meal (17). Moreover, in studies where the meal composition was similar to the FDA meal, the intralumenal environment was only partly characterized (11,13,18). In addition to the buffer capacity, few or no data for osmolality and surface tension data in the fed state have been reported in the literature. Finally, in some studies, agents that induce the fed state rather than an actual meal have been administered to induce "fed state" conditions (19,20). This would minimize the intralumenal volume generated and, as a result, the intralumenal concentrations may be exaggerated compared to those generated during a BA/BE study.

In this study we characterized the upper gastrointestinal (GI) contents under conditions simulating BA/BE studies both in the fasted and in the fed states with a view toward designing media for the *in vitro* study of food effects on dosage form performance. In doing so, we gave emphasis to parameters that have not been fully characterized in the past and/or which are expected to vary with the composition of the administered meal. Fasted state conditions were simulated by administering 250 mL of water to fasted subjects, whereas fed state conditions were simulated by administration of 500 mL of Ensure plus® (21,22). It has previously been shown that Ensure plus® has a similar composition to that of the FDA meal that is commonly administered to study food effects in BA/BE studies (22).

MATERIALS AND METHODS

Phases of the Study

The study consisted of four phases. Each subject was administered a specific volume of fluid in the stomach and samples were aspirated either from the stomach or from a location lower than the sphincter of Oddi in the duodenum as follows:

Phase 1: Samples were aspirated from the antrum of stomach, after administration of 250 mL of water to the antrum through a nasogastric tube.

Phase 2: Samples were aspirated from the antrum of stomach, after administration of 500 mL of Ensure plus® (21,22) to the antrum through a nasogastric tube.

Phase 3: Samples were aspirated from the duodenum after administration of 250 mL of water to the antrum through a nasogastric tube.

Phase 4: Samples were aspirated from the duodenum after administration of 500 mL of Ensure plus® (21) to the antrum through a nasogastric tube.

Subjects

Twenty healthy nonsmokers (16 males and 4 females) with a mean age of 25 years (range 20–32 years) gave informed consent and participated in the study. One subject was 24% heavier than his ideal body weight [as determined from the Metropolitan Life Tables (23)]. Body weights of all other subjects deviated from the ideal weights by less than 10%. None of the participants had a history or any clinical evidence of gastrointestinal disease. The health status of each subject was confirmed by physical examination and screening of blood parameters for renal and hepatic functions.

The study was held in the Red Cross Hospital of Athens after receiving approval by the Scientific and the Executive Committee of the Hospital. The study followed the tenets of the Declarations of Helsinki promulgated in 1964.

From the 80 phases initially planned (20 subjects × 4 phases per subject), a total of 62 phases were successfully completed.

Four phases were incomplete for the following reasons:

– subject vomited during the aspiration period (two phases; one 45 min and another 33 min after administration of Ensure Plus®);

– movement of the tube toward the stomach (two phases; one 40 min and another 100 min after administration of Ensure Plus®).

Fourteen phases were not been performed for the following reasons:

– failure to position the duodenal lumen within a reasonable period (approximately 15 min) (7 phases);

– failure to aspirate samples from the duodenum partly due to creation of a vacuum in the duodenum (two phases);

– subject's decision to terminate his/her participation in the study (five phases).

Study Protocol

The study was performed on two separate experimental days in each subject. Alcohol and any over-the-counter medication were discontinued 3 days prior to and throughout each experimental day, whereas food intake was discontinued for at least 12 h prior to the start of each experimental day and water was restricted on the morning of the experimental day. At about 8 AM on the experimental day, the subject arrived at the clinic and, after a brief screening of his/her health status by a physician, the upper throat was sprayed with lidocaine.

Experimental Day A

The subject was intubated nasally using a sterile disposable tube (Levin #14). The tube is approximately 120 cm long with an external diameter of 4.9 mm. The tube was placed in the antrum of the stomach (under fluoroscopic guidance) and used for both manual administration of meals and manual aspiration of samples. Two hundred and fifty milliliters of mineral water containing 10 mg mL^{-1} PEG 4000 as a nonabsorbable marker were administered through the tube and ~20-mL samples were drawn and placed immediately on ice every 20 min for 60 min. Ninety minutes after water administration, 500 mL of Ensure Plus® containing 10 mg mL^{-1} PEG 4000 were administered to the antrum through the tube, and ~20-mL samples were drawn and placed immediately on ice every 30 min for 210 min. Immediately after each sample was taken, 20 mL of air was pumped into the sampling tube to clear the contents back into the lumen (total internal volume of the tube was estimated to be ~13 mL). After the last sample and before removing the tube/discharging the subject, the position of the tube was confirmed fluoroscopically.

Experimental Day B

The subject was intubated nasally using a sterile two lumen duodenal tube (model 455400 ch.15.0 Ruesch, Stuttgart, Germany). In contrast to some previous aspiration studies [e.g., (14)], no attempt was made to isolate the aspiration segment from the rest of the GI contents. The two lumen tubes were approximately 150 cm long with an external diameter of 4.7 mm and a metal tip at its distal end. A series of holes 27–36 cm proximal to the metal tip was used to access the antrum of the stomach. A further series of holes 0–10 cm proximally to the metal tip was used to aspirate samples from the duodenum (near the ligament of Treitz). Insertion of the tube was assisted by a hydrophilic guiding wire and its position was monitored fluoroscopically. After reaching its final position and removing the wire, 250 mL of mineral water containing 10 mg mL^{-1} PEG 4000 as a nonabsorbable marker were administered using 60-mL (capacity) syringes to the antrum. Thirty minutes after administration of water a ~20-mL sample from the duodenum was aspirated over ice. One hour after administration of water, 500 mL of Ensure Plus® containing 10 mg mL^{-1} PEG 4000 were administered to the antrum using 60-mL syringes over a period of 8–10 min. Samples of up to 20 mL were aspirated over ice from the duodenum every 30 min for 210 min after completion of administration of Ensure plus®. Immediately after each sample was taken, 20 mL of air was pumped into the tube to clear its contents back into the lumen (total internal volume of this sampling tube was estimated to be ~18 mL). At the end of the experimental day and before removing the tube/discharging the subject, the final position of the tube was confirmed fluoroscopically.

Handling and Analysis of Samples

Each aspirated sample was immediately divided into several subsamples and each subsample was used for measuring just one parameter.

pH and buffer capacity measurements were performed on the first subsample immediately upon aspiration. pH values were measured by a pH electrode (ER350B, Metrohm, Herisau, Switzerland). Because of subsample volume restrictions, buffer capacities were measured in just one pH direction, by dropwise addition of either NaOH (samples from fasted stomach) or HCl (samples from fed stomach, fasted duodenum, and fed duodenum). It is worth mentioning that titrating FaSSIF or FeSSIF with HCl has indeed proven to be more appropriate than titrating with NaOH (24). Buffer capacity was calculated according to the following definition: the sample has a buffer capacity value of 1 when one equivalent of strong acid or alkali is required to change the pH value of 1 L by one pH unit (25,26).

Effect of Sample Handling on pH Results. The pH of some subsamples was also measured after maintaining the sample at room temperature without stirring for up to 20 min, to determine whether any drift in the value with time/exposure to open air occurs.

Protein Content: Immediately upon aspiration and before storage at −70°C, gastric subsamples in which total protein content was to be measured were titrated to pH 1 to inhibit proteolytic activity of pepsin (27). Similarly, in duodenal aspirates phenylmethylsulfonyl fluoride (PMSF) was added to inhibit trypsin activity by achieving an end concentration of 1 mM (28). Total protein content was determined using a commercially available kit (BCA, Protein Assay Reagent Kit; Pierce, Rockford, IL, USA) and albumin as a standard. The quantification limit was calculated (29) every analytical day and it was always less than 0.300 mg mL^{-1}.

Pepsin Activity: Immediately upon collection and before storage at −70°C, gastric subsamples in which pepsin activity was to be measured were titrated to pH 6 (27). Pepsin activity was measured by a modification of the method described by Anson (30), and quantification was based on hog pepsin as a standard. The quantification limit was calculated (29) to be 0.010 mg mL^{-1}.

All the remaining subsamples were stored at −70°C immediately after collection. Surface tension was measured using the DeNouy ring method (Sigma70, KSV Instruments, Monroe, CT, USA). Osmolality was measured by using the freezing point depression technique (semimicro osmometer Typ Dig L; Knauer, Berlin, Germany). Total 3α-hydroxy bile acid levels were determined using a commercially available kit (Enzabile; Nycomed, Lidingö, Sweden) and the quantification limit (29) was 500 μM. This kit should only be used for assaying 3α-hydroxy bile salts in simple aqueous samples and/or nonprotein based media after appropriate dilution of the sample with equine serum (which does not contain any bile salts) [e.g., (31)]. PEG 4000 was determined by the method described by Malawer and Powell (32) and modified by Buxton *et al.* (33). The quantification limit (29) of the polyethylene glycol (PEG) assay method was 3.33 mg mL^{-1}. Total carbohydrate content was determined by a modification of the method described by Galanos and Kapoulas (34), using glucose as a standard. The quantification limit (29) was 0.800 mg mL^{-1}.

The physicochemical characteristics of the administered meals, determined using the analytical techniques described above and information from the manufacturers, are presented in Table I.

Data Analysis

Data are presented as box plots showing the median value, the 10th, 25th, 75th, and 90th percentiles, and the

Table I. Physicochemical Characteristics of Administered Liquid Meals

	Water containing 10 mg mL^{-1} PEG	Ensure Plus® containing 10 mg mL^{-1} PEG
Volume (mL)	250	500
Calories (kcal)	0	750
Osmolality (mOsm kg^{-1})	16	610
pH	7.8	6.6
Buffer capacity (mmol L^{-1} ΔpH^{-1})	1	24
Surface tension (mN m^{-1})	62.0	42.4
Total proteins (mg mL^{-1})	–	62
Carbohydrates (mg mL^{-1})	–	202
Fat (mg mL^{-1})	–	49.2

outlier data points, with triangles indicating the mean value. The number of subjects that contributed to a specific box plot is indicated in parentheses above/below each box. Data from a minimum of four subjects was used as the basis for constructing a box plot. Only the data exceeding the quantification limit (LOQ) have been included in the box plots. Data biased to higher values because the number of samples less than LOQ was equal to or greater than the number of samples with greater than LOQ are clearly designated in the text. For each parameter, differences between times were evaluated with one-way ANOVA or the Kruskal–Wallis test. When data did not vary with time, differences between pooled fasted data and pooled fed data were performed with the unpaired *t* test or the Mann–Whitney test. Decision on the use of a parametric or a distribution-free test was made on the basis of the normality and the equal variance tests. Comparisons of pH data were always made with distribution-free tests. All statistical comparisons were performed using Sigmastat 2.03 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Fasted Stomach

Data measured in the fasted stomach are presented in Fig. 1.

Polyethylene Glycol: Median PEG concentration 20 min after administration of water was only 40% of the input value (4.0 mg mL^{-1}). Because the number of samples with PEG concentrations <LOQ was the same as the number of samples having values >LOQ (nine, Fig. 1), data are biased to higher values. Therefore, at 20 min, at least 60% of the contents must have consisted of secretions. Because resting volumes are of the order of 25 mL (35), this rather substantial dilution is attributed to secretions by the gastric mucosa, incoming saliva, and, possibly, incoming nasal secretions generated by the presence of the tube into the nostrils and/or the pharynx (36). Due to sample volume limitations, data at later time points were not collected. The dilution of contents

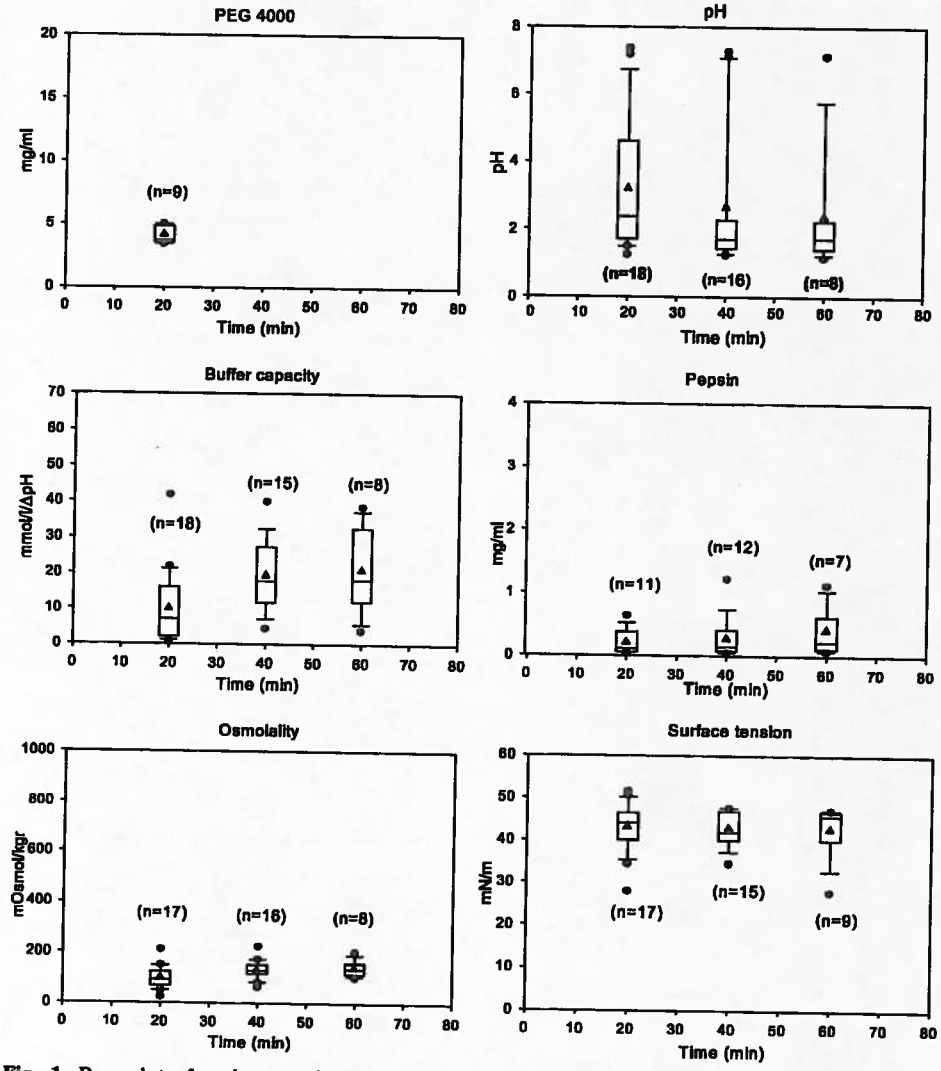

Fig. 1. Box plots for the samples aspirated from the antrum of fasted healthy subjects after administration of 250 mL water containing 10 mg mL^{-1} PEG 4000 into the antrum.

within the 20-min period after administration of water had an impact on pH, buffer capacity, pepsin levels, and osmolality.

pH: Intersubject variation was high (range of individual pH values was 1.23–7.36). Extreme high pH values may in some cases reflect an underlying hypochlorhydria [two subjects in our study consistently showed (at all sampling times) pH values close to neutral], but in most cases they probably reflect the dilution of gastric contents with saliva and/or nasal secretions [the baseline pH of saliva ranges from 5.45 to 6.06 and upon stimulation, the pH rises by about two pH units to a maximum of 7.8 (37)]. Median pH value was 2.4 twenty minutes after administration of water and stabilized to 1.7 at later time points. However, the decline over time did not achieve statistical significance ($p = 0.223$). pH values of 1.7 at late time points are in agreement with the generally accepted value for fasting gastric pH, which is usually measured to be

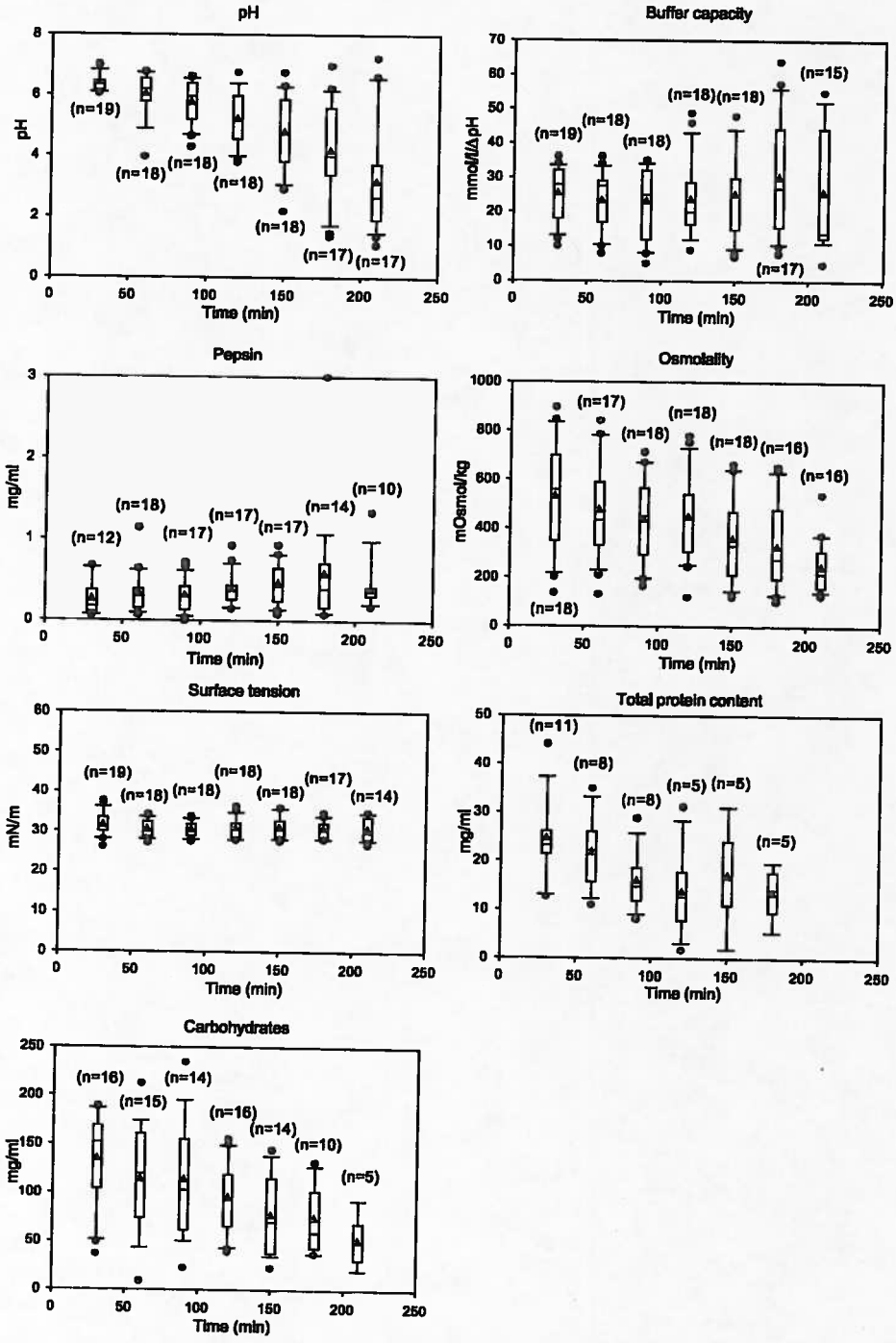

Fig. 2. Box plots for the samples aspirated from the antrum of fasted healthy subjects after administration of 500 mL Ensure Plus® containing 10 mg mL^{-1} PEG 4000 into the antrum.

about 2 or slightly lower (12–17). The pH of fasting gastric aspirates remained unchanged when the samples were maintained at room temperature for up to 20 min.

Buffer Capacity: The median value for buffer capacity 20 min after administration of water was 7 mmol L^{-1} ΔpH^{-1} and increased to about 18 mmol L^{-1} ΔpH^{-1} at later time points ($p < 0.001$).

Pepsin: Median values ranged from 0.11 mg mL^{-1} at 20 min to 0.22 mg mL^{-1} at 60 min (NS, $p > 0.05$). In the literature, values reported for pepsin concentration are higher. Schmidt *et al.* (19) report a value of 0.87 mg mL^{-1} (determined by hydrolysis of hemoglobin at pH 1.7 and by using hog pepsin as standard) and Lambert *et al.* (38) reported values of 0.83–1.27 mg mL^{-1} (determined with an analytical technique similar to that used in this study). An important methodological difference between the present and the previous studies is that no water seems to have been administered prior to collecting aspirates in the previous studies (19,38).

Osmolality: Although gastric contents were clearly hyposmotic, osmolality was lower at early time points (98 mOsm kg^{-1} at 20 min) and plateaued to about 140 mOsm kg^{-1} at later times ($p = 0.026$). These values are consistent with those reported earlier by Gisolfi *et al.* (39) (mean of 29 mOsm kg^{-1} over an 85-min exercise period during which 1,850 mL water was concurrently administered), by Lindahl *et al.* (14) (191 mOsm kg^{-1}, without prior water administration), and by Davenport (37) (171–276 mOsm kg^{-1}, no indication of whether water was preadministered).

Surface Tension: Surface tension was practically unaffected by water administration, with median values ranging from 41.9 to 45.7 mN m^{-1} during the first hour after the administration of water. These values are similar to previously reported results (40,41). Some investigators have attributed the low surface tension of gastric contents (pure water has a surface tension of 72 mN m^{-1}) to a reflux of duodenal contents (42,43). However, others have shown that this cannot be the reason in all subjects, as in many of them the bile salt levels in gastric aspirates are below the limits of detection (41,44–46). It is interesting to note that pepsin alone (at physiological relevant concentrations) is able to decrease the surface tension of water to about 57 mN m^{-1} (4).

Bile Acids: Bile acids, if any, were present at concentrations below the quantification limit of analytical method used in this study (i.e., less than 500 μM). However, bile salts at concentrations up to 1 mM (refluxed from the duodenum) have been quantified by other research groups in the fasted stomach (14,41,43,47). It is worth mentioning that in some of the earlier studies, samples were aspirated from the resting gastric contents rather than during gastric emptying of water from the stomach. From convectional considerations, one might reasonably expect that duodenal reflux would be more pronounced during resting conditions than during active gastric emptying of a liquid.

Fed Stomach

Data for the fed stomach are presented in Fig. 2. In agreement with literature data (33), the analytical method employed in our study for measuring PEG levels was not reliable in the presence of high nutrient concentrations. Therefore, an accurate picture of dilution or concentration of gastric contents over time in the fed state was not possible.

pH: Thirty minutes post-Ensure Plus® administration, the median gastric pH was 6.4 and intersubject variability was low. This value is close to the pH value of Ensure Plus® (6.6). Although intersubject variability increased with time, median pH values gradually decreased ($p < 0.001$) to reach 2.7 at 210 min, indicating that the meal effects on intragastric pH were still apparent 3 h and 30 min after the meal was given. The time required to restore the fasting pH levels depends mainly on the composition and the quantity of the meal, whereas the input pH value seems to be of secondary importance. For example, the time for gastric pH to return to fasting levels after administration of 580 mL of a pH 5.6 meal (651 mOsm, 1,000 kcal) was about 2 h (13), whereas after administration of 400 mL of a pH 6 meal (540 mOsm, 458 kcal) it was about 1 h (11). pH of gastric aspirates drifted in both directions by 5–25% within 15 min when the samples were kept at room temperature.

Buffer Capacity: As with pH, variability increased with time. Unlike pH, no trend in the median value was apparent. During the 30- to 210-min sampling period, median values of buffer capacity ranged from 14 to 28 mmol L^{-1} ΔpH^{-1}, close to input value (Table I). These values are significantly higher than the values measured 20 min after administration of water ($p < 0.001$), but are not different from values measured at times longer then 20 min after the administration of water. Higher total buffer content of gastric contents in the fed compared to the fasting state has also been reported by others; when 10 mL of homogenized meal (500 mL, 546 kcal, ~50% from lipids) was incubated with 20 mL of fresh gastric juice, a 33% increase in buffer capacity in 2 h was generated (48).

Pepsin: Both median values and intersubject variability remained fairly constant with time. During the 30- to 210-min sampling period, pepsin levels ranged from 0.26 to 0.58 mg mL^{-1}. These values are significantly different ($p = 0.006$) and up to twice as high as those measured in fasted state (Fig. 1). However, they are lower than the values found in the study of Lambert *et al.* (38), where pepsin levels in gastric aspirates after intravenous administration of insulin or betazole (histalog) were reported to be 0.56–1.72 mg mL^{-1} and also lower than the values found in the study of Schmidt *et al.* (19), where values of 1.25 and 1.68 mg mL^{-1} were reported after stimulation with histamine or with insulin, respectively. Again, these differences can be attributed to differences in the study protocols; in the earlier studies, which recruited subjects hospitalized for various disorders, induction of the fed state was performed pharmacologically rather than by administration of a meal, leading to substantially lower intragastric volumes and thus, higher pepsin concentration.

Osmolality: Both the median value and intersubject variability decreased with time after ingestion of the meal. The median value 30 min after the administration of the Ensure Plus® was 559 mOsm kg^{-1}, whereas at 210 min it decreased to 217 mOsm kg^{-1} ($p = 0.001$). Mertz and Poppe (20) reported a range of 262–306 mOsm kg^{-1} for osmolality after intravenous infusion of betazole (an analog of histamine); therefore gastric secretions under fed simulating conditions are isoosmotic or only slightly hyperosmotic and the high osmolality of fed aspirates in this study can be attributed to the hyperosmolarity of the administered meal.

Surface Tension: Surface tension values showed remarkable reproducibility and during the entire aspiration period median values ranged from 30 to 31 mN m^{-1}, i.e., they were 30% lower than in the fasting state ($p < 0.001$).

Bile Salts: Only one sample had a bile content above the quantification limit (i.e., higher than 500 μM). In the literature trace levels of bile salts have been reported to be present in the fed stomach [mean value = 60 μM (43)]. Because of the high limit of quantification of our method, it is not surprising that no bile salts could be detected in the stomach either fasted or fed and it is not possible from our results to say whether bile salts are refluxed or not.

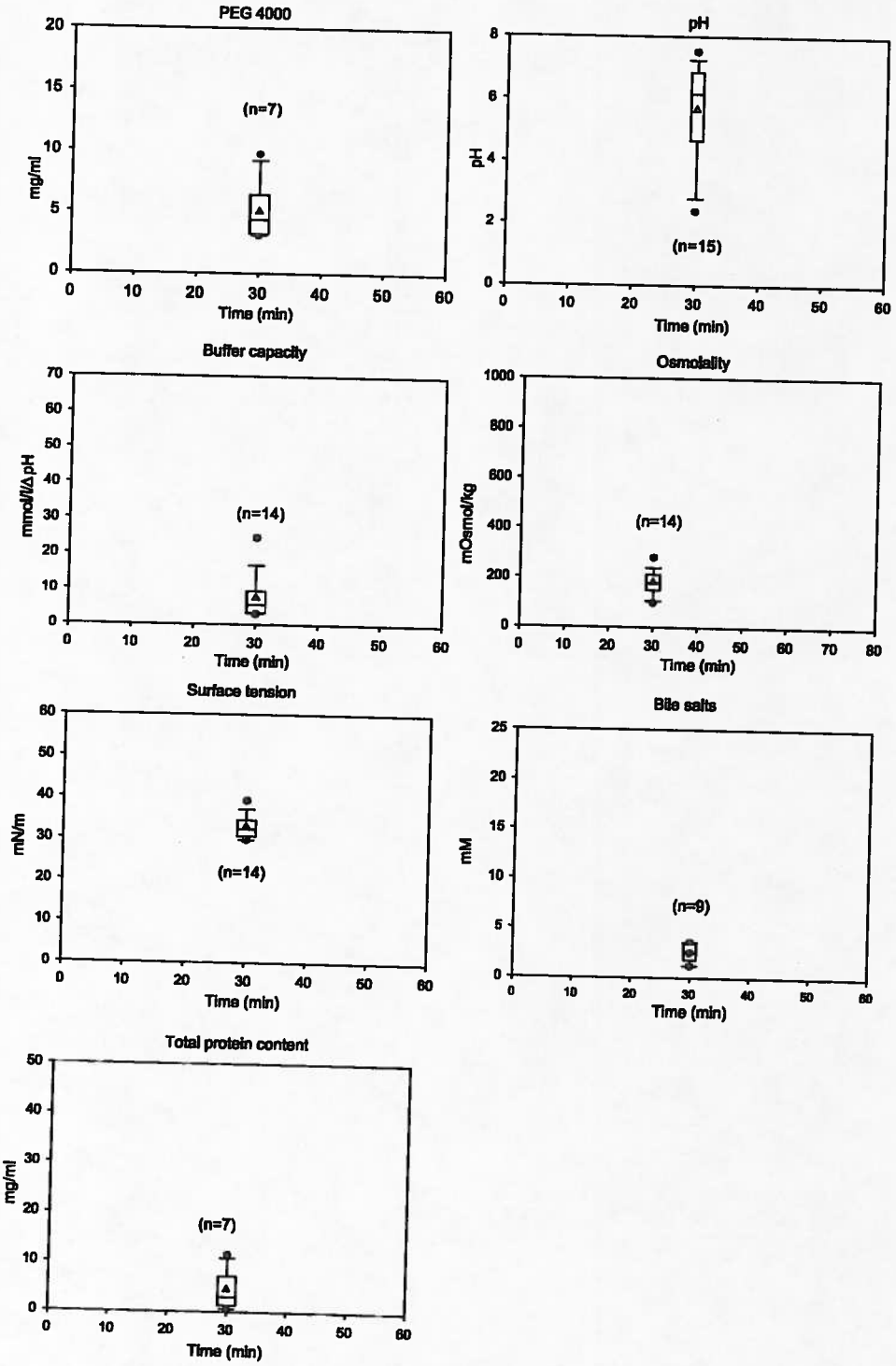

Fig. 3. Box plots for the samples aspirated from the distal duodenum of fasted healthy subjects after administration of 250 mL water containing 10 mg mL^{-1} PEG 4000 into the antrum.

Total Protein and Carbohydrate Content: The median values for total protein and total carbohydrate content decreased gradually from 23.3 and 152.1 mg mL^{-1}, respectively, at 30 min to 11.2 and 49.1 mg mL^{-1}, respectively, at 210 min after the meal's administration. The substantial presence of nutrients 210 min after administration of the meal is in accordance with the higher than baseline pH level discussed earlier. However, it should be noted that, in Fig. 2, total protein data are biased to higher values because the total number of samples with values <LOQ (sixty one) was higher

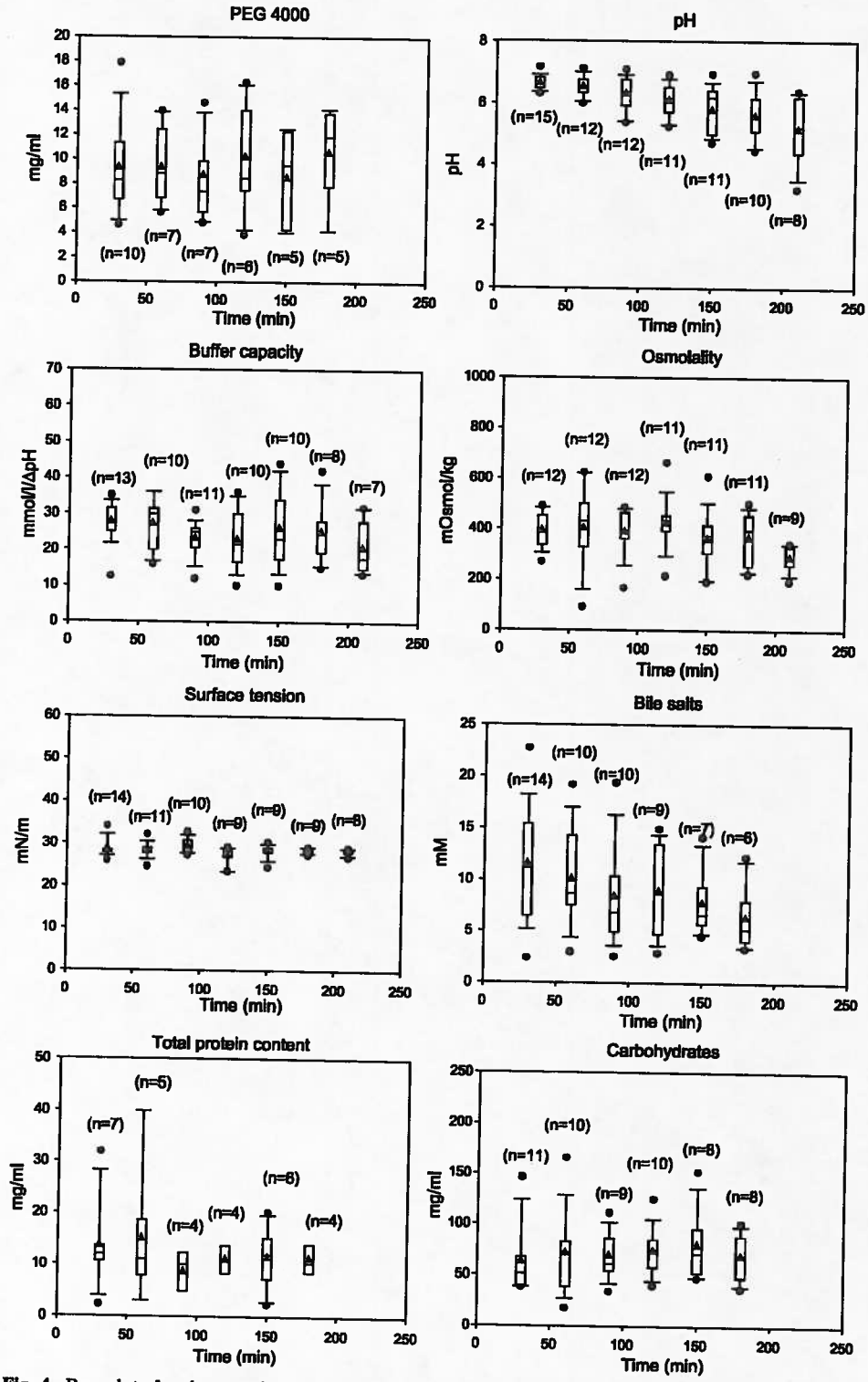

Fig. 4. Box plots for the samples aspirated from the distal duodenum of fasted healthy subjects after administration of 500 mL Ensure Plus® containing 10 mg mL^{-1} PEG 4000 into the antrum.

than the total number of samples with total protein values >LOQ (forty five, Fig. 2).

Fasted Duodenum

Data obtained from duodenal aspirates after administration of water are presented in Fig. 3.

Polyethylene glycol: Median PEG value was 4.3 mg mL^{-1}, similar to the gastric value 20 min after administration of water. These results provide an indication that any water absorption across the duodenal wall is balanced by the baseline bile and pancreatic secretions. However, it should be noted that, in Fig. 3, PEG data are biased to higher values because the total number of samples with values <LOQ (eight) was higher than the total number of samples with total protein values >LOQ (seven, Fig. 3).

pH: As in the fasting stomach, pH values were highly variable. However, the variability observed in the fasted duodenum may be related to reasons other than those speculated for the fasting stomach. According to Woodtli and Owyang (49), intersubject variations of the pH are related to the different phases of interdigestive motility. Moreover, in our study it was confirmed that the two subjects with the lowest intraduodenal pH values were those for which the tube was slightly moved upwards during the experimentation period and, therefore, aspirated sample corresponded to the descending part of the duodenum (i.e., close to or even proximal to the sphincter of Oddi). At the other extreme end, the subject with a slightly alkaline pH in the duodenum was the one that showed almost neutral gastric pH in the fasting state. The median pH value was 6.2. This is in agreement with median fasting duodenal pH values reported in the literature, which vary from 5.95 to 6.72 (10–13,15,50,51). pH of duodenal aspirates drifted to higher values, increasing by up to 6% within 20 min at room temperature. This could be related to a slow transformation of bicarbonates to carbon dioxide under zero-convection conditions (52).

Buffer Capacity: Median buffer capacity was 5.6 mmol L^{-1} ΔpH^{-1}, i.e., much lower than the median gastric value. To the best of our knowledge, there is only one relevant study in which the concentration of bicarbonates immediately next to the duodenal bulb was (albeit indirectly) measured. By measuring pH and partial pressure of carbon dioxide (53), the concentration was found to be about 6.7 mM.

Osmolality: Contents were hypoosmotic (median value = 178 mOsm kg^{-1}). Data are in accordance with those reported by Gisolfi *et al.* (39) (142 mOsm kg^{-1} in intestinal fluids aspirated 25 cm from pylorus) and, as would be expected, lower than the value reported by Lindahl *et al.* (14) for jejunal aspirates (271 mOsm kg^{-1}).

Surface Tension: As in the fasted stomach, this parameter showed the least variability. However, the median value (32.3 mN m^{-1}) was much lower than the gastric value, presumably as a result of the higher level of surface active agents such as bile salts and enzymes.

Bile Salts: The median value was 2.6 mM, similar to the value reported by Lindahl *et al.* (14) for the concentration of bile salts in jejunum during fasted state conditions (average 2.9 mM), but lower than the values found by other investigators for fasted duodenal contents [4.3–6.4 mM (5)].

Total Protein Content: The median value was 3.1 mg mL^{-1} and corresponds to enzymes arriving into duodenum from the stomach (e.g., pepsin, data not shown), the pancreas, and the bile duct [10 g of protein are secreted by the liver into the bile every day in a 70-kg man (54)]. However, this value probably overestimates the actual average intralumenal total protein content because the number of samples with values >LOQ (seven, Fig. 3) was only slightly higher than the number samples with total protein values <LOQ (six). It should be noted that the average total protein content of fasted jejunum has been reported to be 2.1 mg mL^{-1} (14).

Fed Duodenum

Data for the characterization of the luminal contents in the fed duodenum are presented in Fig. 4.

Polyethylene Glycol: Although median values were close to input PEG concentrations, data were extremely variable, suggesting that in some cases there was significant water absorption whereas in others there was significant water secretion. Postprandial values were on the average significantly higher than those 20 min after water administration ($p = 0.006$).

pH: Data were less variable than in the fasting state. The median duodenal pH 30 min after meal administration was 6.6, somewhat higher than the fasting state value, but it fell ($p < 0.001$) slowly to 5.2 at 210 min after the administration of Ensure Plus®. Although the pH decrease with time in the fed upper small intestine is known (10,11), earlier data had suggested that it occurs faster than in the present study and that perhaps the return to the higher pH levels of the fasted duodenal lumen was also faster (11). In the latter study, the energy content of the meal was 458 kcal, with 40% of calories coming from carbohydrates, 20% from proteins, and 40% from fats (11). The meal administered in the present study had similar percentage of calories coming from proteins (the major buffering species among nutrients), but contained much higher total energy content (Table I). Therefore, the buffer capacity is expected to be higher in this study and this could possibly account for the different timescale of progression of the pH value. The pH of duodenal aspirates drifted slightly by up to 3% to lower values within 10 min of storage at room temperature. This is speculated to be related to the creation of digestion products with acidic properties (e.g., digestion of triglycerides).

Buffer Capacity: Median values were between 18 and 30 mmol L^{-1} ΔpH^{-1} without showing a specific trend over time. These values are significantly higher than those measured after water administration ($p < 0.001$). Based on medians, the picture is similar to the corresponding gastric data in the fed state (Fig. 2). It is interesting to note that the extremes in buffer capacity results (high and low) corresponded with the extreme PEG values, i.e., with extremes in net water flux behavior. However, variability in net water flux did not impact the intraduodenal pH (as discussed above), presumably because intraduodenal pH values, the pH of the meal, and the pH of secretions are all close to neutral. Literature data on buffer capacity in the fed duodenum are very limited. Rune (55) measured the pH and the partial

pressure of carbon dioxide in samples aspirated 10 cm lower than the pylorus 3 and 3.75 h after administration of a meal (393 kcal with 39% fats and 51% carbohydrates); estimated bicarbonate concentrations were 10 and 23 mEq L^{-1} (55).

Osmolality: As with both the PEG and the buffer capacity data, osmolality data showed increased variability. The increased variability may be related to the extremes in net water flux observed in some cases and would support the diverse clinical data with regard to the effects of input osmolality on intralumenal water absorption and secretion (39,56–60) that have been reported in the literature. Based on median values, duodenal contents were hyperosmotic over most of the aspiration period but achieved isoosmolality (287 mOsm kg^{-1}) first at 210 min. Values were significantly higher than those after water administration ($p < 0.001$). Ensure plus® contains a disaccharide (25% of total carbohydrate is sucrose), and, like the FDA meal, complex carbohydrates (34% of total carbohydrate is maltodextrin). Gradual hydrolysis of carbohydrates and, perhaps, increased intestinal residence prior to their absorption [41% of total carbohydrates is corn syrup that contains mainly fructose; fructose is absorbed three to six times slower than glucose from the gut (36)] generate higher luminal osmolality (54).

Surface Tension: Surface tension, as in all previous phases, showed the least variability. Medians were very low and ranged between 28.1 and 28.8 mN m^{-1}. Values were significantly different from those measured after water administration ($p < 0.001$).

Bile Salts: Data showed higher variability in the fed state, but results tended to decrease and become more consistent with time. Extreme low values were associated with sampling from the upper-middle duodenum, i.e., close to or even proximal to the sphincter of Oddi. Medians dropped from 11.2 to 5.2 mM at 180 min postdosing. Armand *et al.* (61) reported mean values of 6.7–13.4 mM up to 4 h after a 960-kcal meal (67.5% of calories were from lipids). Fausa (62) reported a mean concentration of 14.5 mM for bile salts at 30 min after administration of the meal (300 mL) and 5.2 mM between 30 and 60 min after administration of the meal.

Total Protein and Carbohydrate Content: Total protein content and total carbohydrate content were variable but much lower than input values over the entire aspiration period. However, data for total protein content are probably biased to higher values because the total number of samples with values <LOQ (twenty nine) was only slightly less than the number of samples with values >LOQ (thirty, Fig. 4). The high total protein content even 180 min after administration of the meal, significantly different than the content measured after water administration ($p = 0.005$), can be attributed partly to the increased presence of enzymes and partly to the presence of proteins in Ensure plus®. Caseinates, as well as other phosphopeptides that are present both in Ensure plus® and in the meal administered in BA/BE studies, are known to be relatively resistant to enzymatic digestion, and their digestibility may be affected by the presence of starch (54). With regard to carbohydrates, significant amounts were still present 180 min after administration of the meal. Although some carbohydrates may be contributed by the bile (37,53), a part of the carbohydrates measured would have been contributed by the maltodextrins [i.e., degradation products of starch; 2–20% of dietary starch and, perhaps, fructose escape absorption in the small bowel (64–66)]. It is worth mentioning that the FDA meal, which is often administered in BA/BE studies, also contains fructose (in orange juice) and starch.

CONCLUSION

The foregoing results confirm that there are very substantial differences in well-characterized parameters such as pH and bile salt concentrations between the fasted and fed states. Furthermore, substantial differences were established in less well-characterized, but pharmaceutically important, parameters such as buffer capacity, osmolality, and volume of luminal contents. A key difference between this study and previous studies was the attempt to simulate usual dosing conditions in a bioavailability/bioequivalence study. For experimental reasons (potential for clogging of aspiration tubes), it was not possible to aspirate after administration of the standard FDA meal. However, a fluid "total nutrition drink" with very similar carbohydrate/protein/fat ratios as well as most other physical chemical properties to the standard meal was substituted, making it possible to aspirate and still at least approximate the conditions usually adopted in bioavailability/bioequivalence studies. In addition, there have been very few studies published that have attempted to characterize luminal conditions in the duodenum after the administration of a meal. The data reported here suggest that, although the current biorelevant media better simulate the luminal environment much more nearly than standard compendial media, there is still some room for improvement. These results, coupled with a separate set of results characterizing the lipids in the GI tract in the fed state, will be used to design a "second generation" of biorelevant media. Potential uses of these media would be for characterization of solubility, dissolution, and permeability properties of drugs and dosage forms.

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